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Placental MicroRNA Expression in Pregnancies Complicated with Preeclampsia and Intrauterine Growth Restriction

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Abstract

A normally developed placenta is integral to a successful pregnancy. Preeclampsia (PE) and intrauterine growth restriction (IUGR) are two common pregnancy related complications that result from abnormal placental development. Placental microRNAs (miRNAs) have been investigated as potential biomarkers for these complications, but they may also play a role in placental development and pathophysiology by influencing gene expression. The objectives of this study are (i) to utilize next-generation sequencing to determine miRNA and gene expression in human placental (chorionic villous) samples from three distinct patient groups with early-onset (EO) PE, IUGR, or PE + IUGR, and (ii) integration of expression datasets to assess the impact of dysregulated miRNAs on gene expression and trophoblast cell function. Placental tissues were collected from four patient groups (control [N=21], EO-PE [N=20], EO-IUGR [N=18], and EO-PE + IUGR [N=20]), and total RNA was used for miRNA and RNA sequencing. Multiple differential expression analysis programs were used to analyze both expression datasets in each patient group compared to gestational age-matched controls. Inverse correlation analysis and target prediction software were used to identify gene targets. Candidate gene targets identified were confirmed using luciferase assays, and impact of miRNAs on trophoblast function was assessed using proliferation and migration assays in HTR-8/SVneo cells. Analysis revealed miRNAs and genes that are disease-specific, as well as others that are common between disease groups, 6 microRNAs and 22 genes were identified to be differentially expressed in all three patient groups. In addition, integrative analysis between the miRNA and gene expression datasets revealed candidate gene targets for miR-193b-5p and miR-210-5p, two miRNAs that also have an impact on trophoblast cell

functions. Our findings suggest common underlying placental pathologies in EO-PE and EO-IUGR. Dysregulated miRNAs and genes identified in this study provide further evidence for trophoblast dysfunction in these pregnancy complications. Integration of miRNA and RNA profiling in the same three subgroups of pregnancy complications, provides an alternate level of molecular information and is a useful tool to expand our understanding of molecular perturbations in the placenta in early onset diseases.

Keywords

Gene Expression, Intrauterine Growth Restriction, MicroRNA Expression, Next-Generation Sequencing, Placenta, Preeclampsia, Target Prediction, Trophoblast Cells

Co-Authorship Statement

Chapter 2 is co-authored by Dr. Gregory B. Gloor, he assisted with bioinformatic analysis of all sequencing data in the study. Chapter 2 is written by me and modified based on review by Dr. Gregory B. Gloor and Dr. Victor Han.

Immunohistochemical analysis in chapter 3 was conducted by Karen Nygard (Biotron, Western University). All other experimental data were collected and analyzed by me. Chapters 1,3, and 4 were written by me and modified based on review by Dr. Victor Han.

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List of Abbreviations

3′UTR: 3′ Untranslated Region

5-hmC: 5- Hydroxymethylated Cytosine

AEDF: Absent End Diastolic Flow

AGO2: Argonaute 2

APLN: Apelin

BP: Blood Pressure

BPD: Basal Plate Decidua

C14MC: Chromosome 14 MicroRNA Cluster

C19MC: Chromosome 19 MicroRNA Cluster

C3AR1: Complement C3a Receptor 1

CAF1: Chromatin Assembly Factor 1

CCR1/4: C-C Motif Chemokine Receptor 1/4

CpG: Cytosine-Phosphate-Guanine

CSF1: Colony Stimulating Factor 1

CT: Cytotrophoblast

CV: Chorionic Villus/Villi

DAB:3,3`- Diaminobenzidine

DGCR8: DiGeorge Syndrome Critical Region Gene 8

DNMT: DNA Methyltransferases

DOHaD: Developmental Origins of Health and Disease Hypothesis

EFNA3: Ephrin A3

EO: Early-Onset

EVT: Extravillous Trophoblast

FGF13: Fibroblast Growth Factor 13

FLT-1: fms-Related Tyrosine Kinase 1

FSTL3: Follstatin-3

FZD5: Frizzled Class Receptor 5

GA: Gestational Age

GEO: Gene Expression Omnibus

GW182: Glycine Tryptophan Protein of 182 KDa

HAT: Histone Acetyltransferases

HDAC: Histone Deacetylase

HELLP: Hemolysis, Elevated Liver Enzymes, Low Platelet Count

HIF-1α: Hypoxia Inducible Factor 1 alpha

HOXA9: Homeobox A9

HTR-8/SVneo: Human Trophoblast/ Simian Virus Neomycin

ICM: Inner Cell Mass

IHC: Immunohistochemistry

IGF2: Insulin Growth Factor 2

IL12RB2: Interleukin 12 Receptor Subunit Beta 2

INHA: Inhibin Subunit Alpha

IQR: Inter-Quantile Range

ITGAM: Integrin Subunit α_M

IUGR: Intrauterine Growth Restriction

lncRNA: Long Non-Coding RNA

LOI: Loss of Imprinting

MALAT1: Metastasis Associated Lung Adenocarcinoma Transcript 1

miRNA: MicroRNA

mRNA: Messenger RNA

NC: Negative Control

NcRNA: Non-Coding RNA

NGS: Next-Generation Sequencing

NRP2: Neuropilin 2

NTN4: Netrin 4

PABP: Poly-A Binding Protein

PCA: Principal Component Analysis

PE: Preeclampsia

PC: Post Conception

PLAP: Placental Alkaline Phosphatase

PMD: Partially Methylated Domain

pri-miRNA: Primary miRNA

PTGS1: Prostaglandin-Endoperoxide Synthase 1

qRT-PCR: Quantitative Real Time Polymerase Chain Reaction

REDF: Reverse End Diastolic Flow

RISC: RNA-Induced Silencing Complex

SDS-PAGE: Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis

SELE: E-Selectin

SEM: Standard Error of the Mean

SGA: Small-For-Gestational-Age

siRNA: Small Interfering RNA

SCT: Syncytiotrophoblast

TLR7: Toll Like Receptor 7

TYRO3: Tyrosine-Protein Kinase Receptor 3

uNK: Uterine Natural Killer

VAV1: Vav Guanine Nucleotide Exchange Factor 1

VT: Villous Trophoblast

WNT3: Wnt Family Member 3

WST-1: Water Soluble Tetrazolium Salts- 1

Chapter 1

1 Introduction

1.1 The Placenta

1.1.1 Placental Development

Placental development in humans begins shortly after the successful implantation of the blastocyst which occurs 6-7 days post-conception (PC).[1] The blastocyst at this time is composed of the outer trophoblast layer known as the trophectoderm which will form the placenta, and the inner cell mass (ICM) which will form the embryo.[1,2] Following successful blastocyst implantation, the outer trophoblast cell layer undergoes rapid proliferation to form two layers of trophoblast cells, the inner cytotrophoblast (CT) layer and the outer syncytiotrophoblast (SCT) layer.[1-3] The SCT cell layer is formed as a result of the fusion of CT cells and the CT layer becomes the main regenerative cell layer.[1,2] By day 8 PC the syncytiotrophoblastic mass expands over the entire surface of the blastocyst.[1]

During days 8-13 PC spaces within the syncytiotrophoblastic cell mass known as lacunae begin to form and expand.[1,2] Also during this time, CT cells begin to differentiate into either the villous trophoblast (VT) or the extravillous trophoblast (EVT) pathway.[1-4] The villous CT cells fuse to form the SCT layer, which grows to form pillars of SCT cells separated by the lacunae known as trabeculae.[1,2] The extravillous CT cells evaginate the SCT layer building cell columns at the terminal ends that differentiate further into endovascular or interstitial trophoblast.[1-4] Extravillous CT cells invade as much as one

third of the maternal myometrium, this process of trophoblast invasion is important for establishment of maternal blood flow into the utero-placental unit, however blood flow at this time is limited due to "plugging" of maternal arteries by the endovascular CT cells.[1,2] In the EVT pathway, interstitial CT cells further differentiate into placental bed giant cells that promote expansion of the placental site.[1-4] The evagination of the SCT layer by extravillous CT cells marks the early development of chorionic villous structures in the placenta.[1-4]

Chorionic villous development continues with proliferation and fusion of CT cells to expand the SCT layer.[1,2,4] The distal ends of the trabeculae fuse together forming the trophoblastic shell, and the trabeculae become the basis for primitive villous trees, meanwhile the lacunar system begins to transform into the intervillous space.[1] Fetal mesenchymal cells migrate and grow into primary villi transforming them into secondary villi.[1,2,4] Finally, by day 18-21 PC fetal vascularization of the villi commences, as secondary villi further expand into tertiary villi.[1,2,4]

Maternal spiral arteries are "unplugged" at 10-12 weeks of pregnancy and maternal blood flows into the intervillous space increasing oxygen tension from 20 mmHg to 60 mmHg.[1,2,4] The increase in oxygen tension triggers remodeling of maternal spiral arteries by endovascular CT cells, this is important to dilate arteries in order to accommodate for increased blood flow to the feto-placental unit, and to relinquish maternal vasomotor control of spiral arteries.[1-4] The maternal and fetal circulations come into close contact with each other but are separated by 4 main layers, (1) continuous SCT layer that lines the intervillous space; (2) layer of CT cells (attenuates in 2nd and 3rd trimester); (3)

trophoblastic basal lamina; and (4) fetal endothelium.[1,2] Figure 1.1 shows a sagittal section of the mature placenta, as well as a chorionic villus tree, and a cross section of the chorionic villus branch.

1.1.2 Placental Function

During gestation, the placenta along with fetal membranes and the amniotic fluid work together to maintain normal growth and development of the fetus. The placenta has three main important functions: (1) it is the main site of nutrient, oxygen and waste exchange, (2) it also functions as an endocrine tissue that regulates the intrauterine milieu through production and regulation of hormone levels, and (3) it protects the fetus against infection and xenobiotic molecules.[2,5]

In the mature placenta, the fetal aspect is called the chorionic plate, and the maternal aspect is called the basal plate (Figure 1.1a) .[1,2] In between is the intervillous space containing the main functional units of the placenta, the chorionic villi (Figure 1.1a).[1,2,4] At the terminal regions of the chorionic villi is where maternal-fetal exchange occurs, maternal blood containing necessary oxygen and nutrients (carbohydrates, amino acids, lipids, vitamins, minerals) enters the intervillous space via the remodeled spiral arteries, meanwhile oxygen-deficient blood and waste products drain through the endometrial veins (Figure 1.1a).[2,5] As an endocrine tissue, the placenta releases hormones into the maternal and fetal circulation in order to regulate processes such as fetal growth, metabolism, and parturition.[2,5] The placenta produces endocrine, paracrine, and autocrine factors. Examples of these factors include but are not limited to: estrogen, progesterone,

placental growth hormone, and a number of growth factors such as insulin like growth factors I and II.[2,5] As a protective tissue the placenta is equipped with export pumps and enzymes that can metabolize drugs and xenobiotic molecules.[2,5]

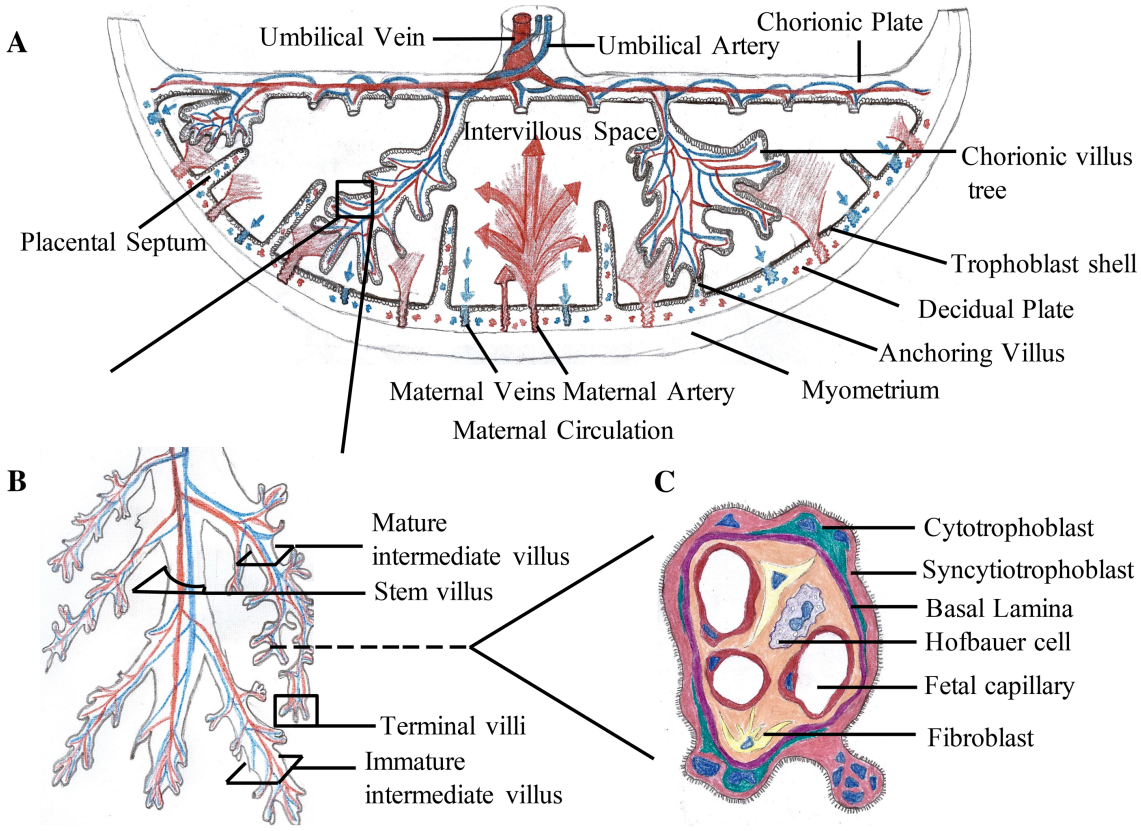

Figure 1.1 Sagittal and cross-sectional views of mature placental structure

A. Sagittal view of the mature placental after complete development in the 2nd trimester, which includes the chorionic plate, the basal plate decidua, and **B**. the main functional unit of the placenta- the chorionic villus tree within the intervillous space. **C**. Cross-section view of the terminal villi showing layers that separate maternal and fetal circulation.

1.1.3 Pregnancy Complications: PE

Preeclampsia (PE) is a maternal pregnancy complication that impacts 2-8% of pregnancies.[6,7] The two hallmarks of PE are (1) maternal systolic pressure greater than 140 mmHg and diastolic pressure greater than 90 mmHg, and (2) 24-hour proteinuria of > 0.3 g; these are the current criteria used for diagnosis of PE.[6,7] There are a number of risk factors that could increase the likelihood of developing PE during pregnancy. Many of those factors are maternal, including maternal diabetes, preexisting hypertension, and obesity.[6-8]

One of the leading hypotheses on the pathogenesis of PE states that this maternal systemic disturbance is due to poor remodeling of maternal spiral arteries by EVT cells (Figure 1.2).[6-9] Extravillous CT cells are responsible for migration, invasion and remodeling of maternal spiral arteries to accommodate for increased blood flow into the feto-placental unit necessary for fetal development.[6-9] If this highly coordinated, complex process doesn't occur properly, it results in shallow and incomplete remodeling of spiral arteries that remain partly under maternal vasomotor control and in poor placental perfusion.[8,9] Poor placental perfusion results in oxidative stress and chronic placental ischemia, this triggers the release of antiangiogenic factors and proinflammatory cytokines from the placenta into maternal circulation resulting in a systemic maternal disturbance during pregnancy.[8,9] Examples of antiangiogenic factors commonly identified in preeclamptic maternal plasma include sFLT-1 and Endoglin.[8,9] On the other hand, the specific mechanisms by which extravillous CT cells fail to properly remodel maternal spiral arteries are currently under investigation.

The only cure for PE currently is the delivery of the fetus and the placenta, therefore both maternal and fetal health are at risk, were chances of preterm birth are higher and adverse effects on maternal health are imminent. Patients are currently classified into early- and late- onset PE depending on onset and severity of symptoms.[6,7] In early-onset patients symptoms appear prior to 34 weeks of gestation and are more likely to be severe, and at times associated with HELLP (Hemolysis, Elevated Liver enzymes, and Low Platelet count).[6,7] In late-onset patients symptoms appear after 34 weeks of gestation and in many cases patients deliver at term or late preterm with proper management of symptoms.[6,7]

1.1.4 Pregnancy Complications: IUGR

Intrauterine growth restriction (IUGR) is characterized by poor fetal growth *in utero*, where estimated weight of the fetus is less than the 10th percentile for gestational age and gender.[10,11] Based on current diagnosis criteria for IUGR, it may occur early in pregnancy (< 32 weeks) or later (> 32 weeks), defined as early- and late-onset of IUGR respectively.[12] It is now known that IUGR and small for gestational age (SGA) are distinct. IUGR refers to pathological asymmetric fetal smallness that is commonly associated with poor feto-placental Dopplers, whereas SGA refers to non-pathological constitutional fetal smallness that is more commonly associated with normal feto-placental Dopplers.[10-12] IUGR is also associated with significant perinatal morbidity and mortality, as well as adverse postnatal outcomes.[10]

There are maternal and fetal risk factors associated with the etiology of IUGR. Similar to PE, maternal risk factors include diabetes, obesity, smoking and alcohol consumption.[10,11]

Fetal risk factors include chromosomal and genetic abnormalities, congenital heart defects, and infection.[10,11] However, in many cases the etiology of IUGR can be attributed to placental insufficiency, an encompassing condition in which the placenta fails to function normally and the pathophysiology of which is not well understood.[10-12] Diagnosing IUGR can be challenging, although biochemical testing in maternal plasma in the first trimester can be used as an indicator of placenta related complications, the sensitivity and specificity of the results are not sufficient for concrete diagnosis of placental dysfunction.[10,11] Feto-placental Doppler ultrasound is currently the most reliable tool for diagnosing IUGR.[12] More specifically, umbilical artery Doppler is used to assess placental function during pregnancy, absent or reverse end diastolic flow (AEDF or REDF) indicates severe placental impairment.[10-12] Poor umbilical artery Dopplers indicative of deteriorating placental function is a finding more commonly associated with early-onset IUGR, and more often necessitates early delivery of the fetus to avoid mortality *in utero*.[10-12]

Early-onset IUGR is also commonly associated with gestational hypertension and PE.[12] The theory of impaired trophoblast invasion to transform maternal spiral arteries resulting in a poorly-perfused and poorly developed placenta has also been associated with IUGR but to a lesser extent.[4,12,13] One consistent finding on the other hand, is the histopathological evidence of placental ischemia and oxidative stress in placentae from both IUGR and PE pregnancies.[13-16] Common histopathological features include: villous infarctions, fibrin deposition, and syncytial knotting.[14-16] This provides evidence for some common underlying pathophysiological mechanisms in the placenta in PE and IUGR.

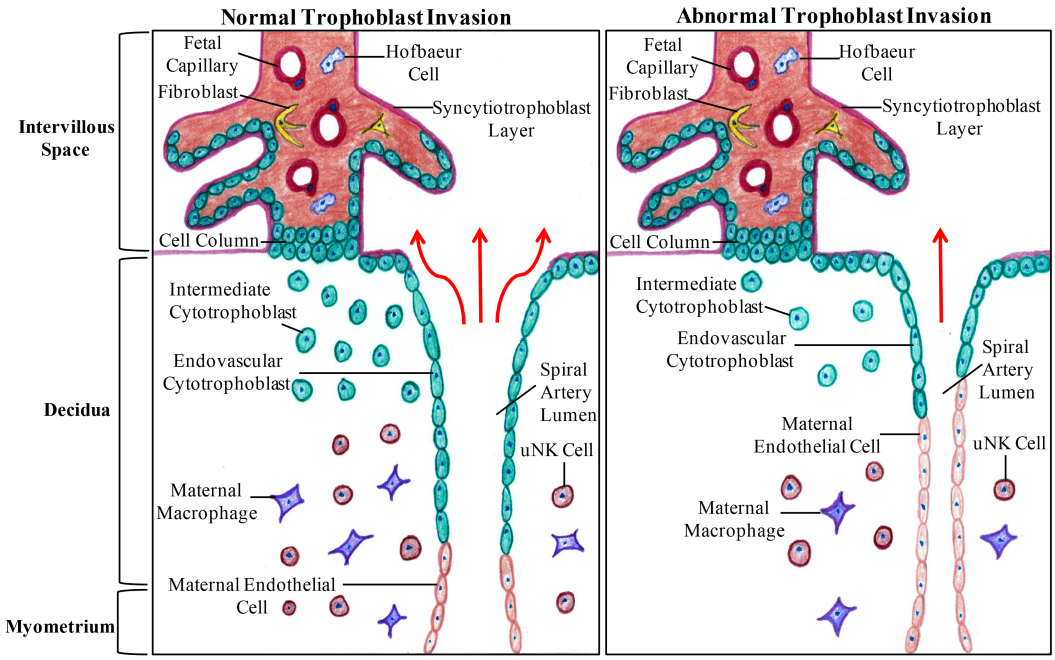

Figure 1.2 Schematic depicting normal and abnormal trophoblast invasion.

Trophoblast invasion is a process necessary to increase maternal blood flow into the uteroplacental unit to support gas and nutrient exchange for pregnancy. The left panel depicts normal trophoblast invasion, where extravillous CT cells differentiate further into endovascular CT cells which remodel maternal spiral arteries by replacing maternal endothelial cells, this also results in dilation of maternal arteries. This process occurs in the presence of maternal immune cells including uterine natural killers (uNK) cells and macrophages. The right panel depicts abnormal trophoblast invasion, represented by failure of trophoblast cells to migrate and invade spiral arteries. Subsequently, spiral arteries remain undilated and under maternal vasomotor control as trophoblast cells fail to replace maternal vascular endothelial cells. This results in decreased perfusion of maternal blood into the intervillous space.

1.2 DOHaD and Epigenetic Regulation

1.2.1 Developmental origins of health and disease hypothesis (DOHaD)

Infants exposed to intrauterine stress during pregnancy, particularly growth restricted and preterm infants are at higher risk for perinatal morbidity and adult onset diseases.[17] This is recognized through the "developmental origins of health and disease" (DOHaD) hypothesis, which states that alterations from a normal environment during critical and sensitive periods of growth and development result in gene expression changes in the fetus, which lead to changes in fetal phenotype including birth weight and predisposition to disease later on in life.[17] Fetal programming is the term used to refer to the genetic and epigenetic changes occurring *in utero* in response to adverse environmental signals.[17,18] Due to the placenta's pivotal role in regulating and supporting fetal growth it is directly involved in fetal programming in early life.[18-20]

Placental dysfunction is a feature of pregnancies that lead to fetal programming effects, most commonly identified in IUGR pregnancies.[18,20] However, programming effects are also observed in preterm births, PE, and uncontrolled gestational diabetes.[18,20] Pathologies in the placenta such as maldevelopment of villous trees, impaired vasculogenesis, poor syncytiotrophoblast repair and accumulation of syncytial knots, directly impact placental function.[18-20] Most importantly pathologies in the placenta will impact nutrient and oxygen delivery to the fetus and the synthesis and regulation of hormones necessary for fetal and placental growth.[19,21] The placenta must also adapt to maternal signals including maternal hormones, nutrition, and BMI.[19,21] Therefore, investigation of genetic and epigenetic changes in the placenta, particularly changes

involved in disease pathophysiology, is important to expand our understanding of the role of the placenta in fetal programming *in utero.*

1.2.2 Epigenetics

Epigenetic mechanisms are mechanisms that regulate gene expression without changes to the underlying DNA sequence.[22,23] The profile of epigenetic modifications of a cell, although often identified through studying the tissue, is identified as the epigenome.[24] Epigenetic marks are also heritable through cell division and are plastic, these characteristics are observed through transgenerational inheritance of epigenetic marks and changes in response to environmental signals.[23,24] Research has identified a number of epigenetic mechanisms including DNA methylation, histone modifications and non-coding RNAs (Figure 1.3).[22,23]

DNA methylation involves the covalent transfer of a methyl group to the C-5 position of the cytosine ring via enzymes called DNA methyltransferases (DNMT).[22,23] Methylation commonly occurs in cytosine-guanidine (CpG) rich regions.[22,23] Regions of the genome with high CpG content are identified as CpG islands and can be found in gene promoter regions.[22,23] Islands associated with gene promoters are important sites of regulation where methylation can result in gene silencing by suppressing transcription, and the reverse, the removal of a methyl group will activate transcription and gene expression.[22,23] In recent studies, the role of hydroxymethylated cytosines (5-hmC) as an epigenetic mark has also been studied.[25] 5-hmC differs from methylated cytosines in that there is an additional hydroxyl group.[25] Initially 5-hmC was thought to be an intermediate

step in demethylation, but now it is hypothesized that in fact it has a role in regulating gene expression.[25] Chromatin is composed of nucleosomes which consist of DNA wrapped around histone proteins.[22,23] The DNA-protein complex is subject to covalent modification via the exposed N-terminal tail of the histone protein.[22,23] Modifications include phosphorylation, methylation, acetylation and ubiquitinylation, and each modification can result in either transcriptional activation or repression.[22,23] Similar to DNA methylation, histone modifications require enzymes called histone transferases.[22] There are well studied histone modifications for transcriptional activation such as histone acetylation or di-/tri-methylation of lysine 4 of histone 3.[22] Meanwhile di-/tri-methylation of lysines 9 or 27 of histone 3 leads to transcriptional repression.[22] Parts of the genome encoding non-coding RNAs (ncRNAs), including miRNAs were once considered junk DNA. Advancements in research later showed these regions of the genome are transcriptionally active but are not translated into proteins. These transcribed ncRNAs were found to be important regulators of many developmental processes. NcRNAs are classified into categories based on length and function. The most common categories are long non-coding RNAs (lncRNA), small interfering RNAs (siRNA) and microRNAs (miRNA) which are RNAs processed from primary transcripts in the nucleus and can alter gene expression by binding to the mRNA of the target gene in the cytoplasm.[22,23] Epigenetic regulation of gene expression in the placenta during this critical period of development plays a direct role in fetal programming.[23,24,26]

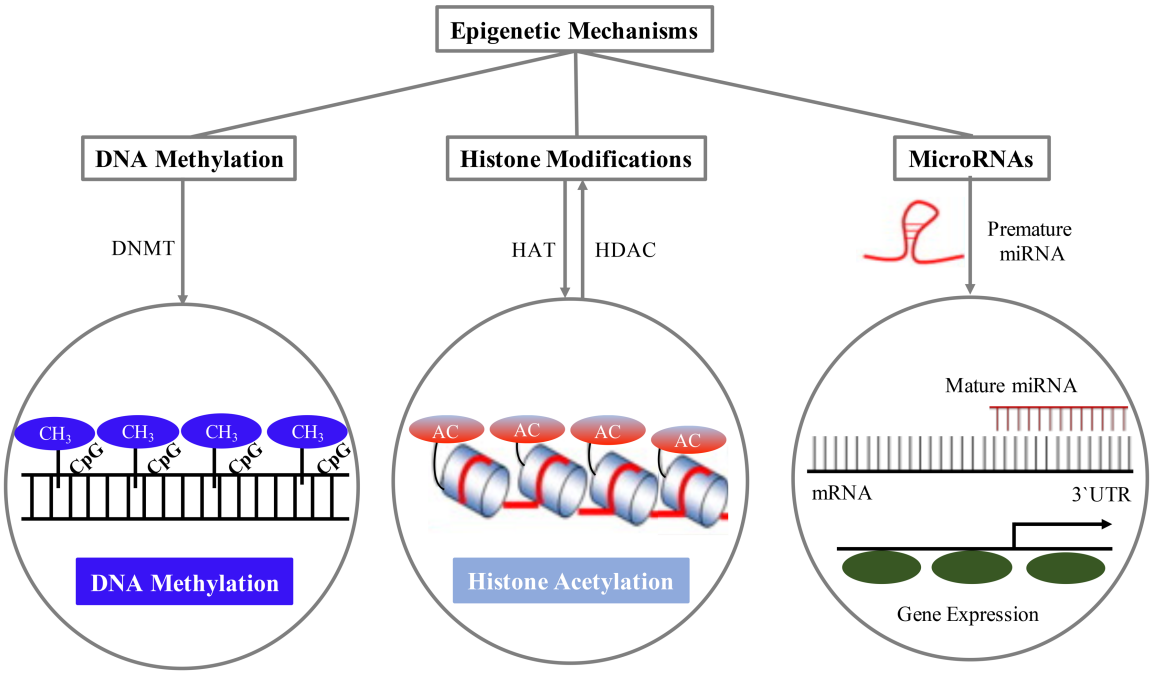

Figure 1.3 Epigenetic mechanisms that can alter gene expression without changes to the underlying DNA sequence.

1.2.3 MicroRNAs

MiRNAs were originally described in the nematode C. elegans, and then later discovered in the genomes of plants and animals.[27] MiRNAs can regulate gene expression by binding to the target mRNA resulting in degradation or halting protein translation.[27,28] The first step in miRNA biogenesis is transcription by RNA polymerase II into a looped, hairpin-shaped, primary miRNA (pri-miRNA).[27,28] A RNase III endonuclease called Drosha enzymatically cleaves pri-miRNA, with guidance from DGCR8 and RNA binding proteins, into a stem-looped precursor miRNA that is 60-70 nucleotides long. Precursor miRNAs are transported into the cytoplasm through exportin 5, as the name suggests an exporter on the nuclear membrane.[27,28] In the cytoplasm, a second cleavage event by

dicer, also an RNase III endonuclease, releases the loop and results in a mature miRNA duplex that is 20-22 nucleotides in length.[27,28] The double stranded miRNA then binds RISC (RNA-induced silencing complex) and is unwound, the guide strand remains attached to RISC and the passenger strand is degraded.[27,28] Argonaute 2 (AGO2) and GW182 are core RISC members and with help of accessory proteins they facilitate miRNA targeting.[27-29]

Complementarity of the miRNA to the mRNA determines the fate of the mRNA. A 6-7 nucleotide region of the miRNA identified as the seed region targets the 3ˈUTR of the mRNA.[30] Interactions between mRNAs and miRNAs at the seed regions have been categorized into three canonical site types (1) 7mer-A1: 6 nucleotide seed match plus adenosine at position 1, (2) 7mer-m8: 6 nucleotide seed match plus a match at position 8 , and (3) 8mer: 6 nucleotide seed match plus a match at position 8 and adenosine at position 1.[30] Perfect complementarity between the miRNA seed region and the 3ˈUTR of the mRNA will target the mRNA for degradation by cleavage.[27-30] This process is mediated through the GW182 recruitment of poly-A binding protein (PABP) which subsequently recruit deadenylases CCR4 and CAF1.[27-30] Mismatch between the miRNA seed region and the 3ˈUTR of the mRNA will result in translational repression through RISC's recruitment of accessory proteins that will prevent translational machinery from translating the mRNA into a protein.[28-30]

MiRNA biogenesis and function are complex processes that involve large networks of proteins that remain under investigation today. For example the miRNA duplex that forms in the cytoplasm as a result of dicer cleavage contains a guide strand and a

passenger strand, and the proper miRNA nomenclature now refers to the two strands as -3p and -5p respectively.[30,31] Although it was once thought that only the -3p strand exerted biological functions, recent evidence shows that the -5p strand can also be the "guide strand".[31] However, the process by which this is regulated remains under investigation. Even with the complexities of these processes, miRNAs have been shown to regulate large numbers of target mRNAs and are estimated to potentially regulate 60% of genes in eukaryotic genomes.[32,33] MiRNAs have also been implicated in diseases such as cancer, diabetes, cardiovascular disease and pregnancy complications.[34]

1.3 Epigenetic regulation in the placenta

1.3.1 DNA methylation

DNA methylation is one of the more extensively investigated epigenetic regulatory mechanisms in the human placenta. The placental methylome has been investigated in the normal human placenta across gestation, in different placental cell types, in pregnancy complications, and in its response to environmental signals.[24,26,35] During the pre-implantation phase, the fertilized ovum undergoes an erasure of methylation marks and by the blastocyst stage, the genome is almost entirely hypomethylated, with the exception of imprinted genes, which are genes expressed mono allelically in a parent-of-origin dependent manner.[36,37] After differentiation into the ICM and the trophectoderm, *de novo* methylation takes place but is restricted to the ICM, while the trophectoderm, which gives rise to the placenta remains virtually hypomethylated.[37,38]

Studies have shown that the placental genome remains hypomethylated compared to somatic tissues and cells.[39,40] Global methylation levels are also shown to increase across gestation, from first to third trimester, however this could potentially be attributed to changes in cell composition in response to progress of gestation.[40,41] A landmark study by Schroeder et al. in 2013, showed that the global hypomethylated profile of the placenta is organized into partially methylated regions (PMDs), which are large contiguous domains (> 100 kb) that have reduced DNA methylation and cover 40% of the placental genome.[42] Genes in the placenta found within PMDs are repressed, as confirmed by RNA-seq, have tissue-specific functions and demonstrate stability across gestation.[42] Interestingly, methylation at promoter CpG islands was more commonly found in the genes that fall within PMDs, which is a possible explanation for the repressed state of these genes.[42] In total, 3,815 genes were identified to overlap PMDs providing evidence for epigenetic regulation of gene expression in the placenta.[42]

Since the placenta is composed of several cell types, it is important to consider that each cell type can have unique epigenetic marks that contribute to the epigenetic profile of the placenta. Indeed, studies have shown differences in DNA methylation between placental CTs and fibroblasts (at 442 CpGs), and in comparing methylation in each cell type to whole placental villi samples.[43] Other studies have shown differences in DNA methylation in CT and SCT cells, with potential functional implications since differentially methylated regions in EVT cells are enriched for genes important for invasion and proliferation.[44-47]

Epigenetic regulation via DNA methylation in human placentae from complicated pregnancies such as PE, IUGR, gestational diabetes and pre-term births is also being extensively researched. In PE pregnancies, one study identified a significant overall increase in global methylation in PE placenta compared to controls.[48] The same study noted a positive association between global methylation levels and maternal systolic/diastolic pressure.[48] PE has also been associated with altered methylation of a subset of genes, including but not limited to, *TIMP53*[49], *SERPINB5*[50] and *SERPINA3*[51,52], and *11βHSD*[53]. In IUGR, an early study identified 22 genes with differential methylation between control and diseased placentae, some with reduced and some with increased methylation.[54] Down-stream analysis revealed those genes are implicated in Huntington's disease, Alzheimer's disease and protein export.[54] A more recent study identified no differentially methylated positions between IUGR placenta and control (term) placenta.[55] The placental epigenome is also plastic, this is evident from studies observing changes in DNA methylation in response to environmental exposures.[56-58] Collectively, these findings provide evidence for the presence of epigenetic regulation via DNA methylation in the human placenta, and its direct role in placental growth and function, and subsequently fetal programming.

1.3.2 Non-coding RNAs

1.3.2.1 Long Non-coding RNAs in the placenta

Long non-coding RNAs (lncRNAs) are widely expressed in the human genome and recent advancements in NGS technology allow for identification of novel lncRNAs implicated in development and disease. lncRNAs are > 200 nucleotides in length and

have a diverse range of molecular functions.[59,60] In the placenta, dysregulation of lncRNAs is reported in PE, IUGR and other placenta related complications.[61-65]

H19 is a commonly investigated lncRNA located within a large imprinted region on chromosome 11, 130 kb downstream of *IGF2*.[66] *H19*'s reported functions include a modulator for binding small RNAs and proteins, and a source of miR-675.[67,68] In the placenta *H19* expression is restricted to the intermediate and villous cytotrophoblasts and is thought to regulate the invasive properties of trophoblast cells.[69,70] In EO-PE placenta the *H19* promoter region is hypermethylated and is correlated with decreased *H19* expression.[63,71] Altered *H19* and *IGF2* expression has also been reported in placenta from IUGR and SGA pregnancies.[63,65,72] In placenta from IUGR pregnancies there is partial loss of imprinting (LOI) with biallelic expression of *H19*.[65] *MALAT1* is another lncRNA with reported decreased expression in PE placenta compared to controls.[62] *MALAT1* expression has been shown to increase invasion and migration in trophoblast cell lines.[62,73] Interestingly *MALAT1* expression is increased in placenta increta and in contrast to shallow invasion in PE, placenta increta is overly invasive.[73] Ongoing discovery and characterization of novel and known lncRNAs is important to increase our understanding of this mechanism in placental development and disease.

1.3.2.2 MicroRNAs in the placenta

MiRNAs are widely expressed in normal human tissues and can exhibit tissue specificity.[74] In the placenta, two miRNA clusters are predominantly expressed and are only conserved in mammals, C19MC and C14MC, chromosome 19 and 14 miRNA

clusters, respectively.[75,76] The C19MC cluster is located at 19q13.41, it spans ~ 100 Kb of genomic DNA, and is one of the larger miRNA clusters identified to date.[75,76] This primate-specific cluster contains 46 pre-miRNAs expressed exclusively from the paternal allele, and produces 58 mature miRNAs.[75,76] Functionally, C19MC expression is reduced in human EVT cells compared to VT cells, and expression of the C19MC attenuated migration of human EVT cells *in vitro*.[77] Directly adjacent to the C19MC is a small cluster known as the miR-371-3 cluster, which is also conserved in mammals and predominantly expressed in the placenta.[75,76] The miR-371-3 cluster includes three miRNAs and spans 1050 bp.[75,76] The C14MC cluster is located at 14q32, it spans ~ 40 Kb of genomic DNA, and it harbors 52 miRNA genes expressed exclusively from the maternal allele, and produces 63 mature miRNAs.[78] Specific functions for the C14MC have not yet been identified in the placenta.

The main source of miRNAs in the placenta is trophoblast cells, therefore ongoing research is focused on the role of miRNAs in trophoblast functions.[76] One example is the miR-17 ~ 92 miRNA cluster that has been shown to regulate trophoblast differentiation by targeting important proteins such as CYP19A1(aromatase) and the transcription factor GCM1.[79] Studies have identified differential expression in miRNAs between first and third trimester control human placenta, alluding to the potential role of miRNAs in placental development.[76,80] A study by Gu et al. (2013), identified 191 mature miRNAs to be differentially expressed between first and third trimester placental villous samples.[80] In addition, miRNAs within the C19MC, C14MC, and the miR-371 clusters are upregulated in first trimester samples compared to third trimester samples.[80] MiRNAs that are highly

expressed in the first trimester are related to angiogenic, oncogenic and anti-apoptotic function, whereas, miRNAs expressed in the third trimester are related to cell differentiation and tumor suppression functions.[80] Interest in investigating the role of placental miRNAs in placental growth, function, and particularly disease was further increased with discovery of placental miRNAs in maternal circulation.[81] A study by Luo et al. (2009) identified exosomes as a mechanism of entry for placenta-specific miRNAs originally expressed in trophoblast cells into maternal circulation.[81]

1.3.2.3 MicroRNAs in maternal circulation

Early studies measuring miRNA expression in maternal plasma utilized cell-free RNA. [82] Later studies show that placenta-specific miRNAs can enter maternal circulation via exosomes, extracellular vesicles 40-120 nm in size involved in cell-cell signaling and communication.[81] Protocols for isolation and purification of trophoblast-specific exosomes from maternal plasma have been challenging and most current studies continue to utilize cell-free RNA. One of the earliest studies conducted by Chim et al. (2008), assessed the expression of 157 miRNAs in maternal plasma from pregnant women, and identified four miRNAs that were highly expressed (miR-141, miR-149, miR-299-5p, miR-135b).[82] Further studies investigated the expression of C19MC in maternal plasma of pregnant women compared with non-pregnant women. Seven miRNAs (miR-516-5p, miR-517, miR-518b, miR-520a, miR-520h, miR-525 and miR-526a) were identified as new pregnancy associated miRNAs with diagnostic potential.[83] Some of these miRNAs have also been identified in maternal plasma by other studies, such as miR-517 and miR-518.[84] However, expression levels of miRNAs in maternal plasma can be confounded by

the mode of delivery. In a study by Morisaki et al. (2015), levels of placenta-specific miRNAs such as miR-515-3p, miR-517a/c, and miR-518b are significantly higher in the patient group that had undergone labor compared to the non-labor group.[85]

More recent studies have focused on the detection of miRNAs in the plasma of women diagnosed with PE and/or IUGR, to identify miRNAs with diagnostic potential. The levels of ten C19MC miRNAs were found to be significantly elevated in maternal plasma from patients with severe PE.[86] In first trimester maternal plasma samples a predictive value for miR-517-5p, miR-518b, and miR-520h with PE was identified.[87] Increased expression of these miRNAs was observed in first trimester plasma samples from women who developed PE later in pregnancy compared to normal pregnancies.[87] The same subset of miRNAs was assessed for their predictive value of IUGR, but no associations were identified.[87] Another study by Hromadnikova et al. (2016) comparing maternal plasma samples from PE and IUGR pregnancies to control pregnancies, identified downregulation of miR-100-5p, miR-125b-5p, and miR-199a-5p.[88] Although many miRNAs have been identified in maternal plasma from PE and IUGR pregnancies, currently, there is no agreed upon subset of miRNAs associated with either PE or IUGR with promising diagnostic potential. This can be attributed to differences in patient populations and approaches to miRNA quantification, however research on the potential use of miRNAs as a diagnostic or predictive tool for pregnancy complications remains ongoing.

1.4 Rationale

The placenta performs important functions during pregnancy and it is key player in fetal growth and development.[1,2,4,5] Pregnancy complications such as PE and IUGR implicate the placenta in disease etiology.[6,7] The placenta must also adapt to maternal stimuli such as poor nutrition, obesity and preexisting medical conditions.[18-21] This directly implicates the placenta in fetal programming.[18,21] There is accumulating evidence for the presence of epigenetic mechanisms in the placental genome, in addition to evidence of differential epigenetic regulation during pregnancy complications that may contribute to fetal programming.[42,48,59,60] This study aims to investigate miRNA and gene expression in a matching sample set of well-defined patient groups with early-onset pregnancy complications to expand our understanding of miRNA function in the placenta in the pathophysiology of diseases with placental origins. Due to the common overlap of PE and IUGR in early-onset patients[12], we segregated patients into 3 groups: PE only, IUGR only, and PE+IUGR to identify similarities and differences.

1.5 Hypothesis and Objectives

MicroRNAs are differentially expressed in diseased placentae obtained from pregnancies complicated by early-onset intrauterine growth restriction (IUGR) and/or preeclampsia (PE) and they regulate downstream gene targets involved in the growth/development and functions of the placenta.

This hypothesis will be addressed in the following 3 objectives:

1. Conduct miRNA expression analysis in placental samples from early-onset (EO) IUGR, EO-PE, as well as pregnancies complicated by both EO-PE and EO-IUGR

2. Conduct gene expression analysis in the same placental samples from EO-IUGR, EO-PE, as well as EO-PE+IUGR, and investigate downstream impact of miRNA dysregulation

3. Transfect a trophoblast cell line with miRNA mimics and inhibitors to investigate the functional impact of miRNAs *in vitro*

1.6 References

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Chapter 2

2 Placental microRNAs in pregnancies with early onset intrauterine growth restriction and preeclampsia: Potential impact on gene expression and pathophysiology[1]

2.1 Introduction

Abnormal placental development in pregnancy may result in complications such as preeclampsia (PE) and intrauterine growth restriction (IUGR).[1,2] PE is a maternal hypertensive disorder occurring in 2-8% of pregnancies worldwide.[3,4] Intrauterine growth restriction is poor fetal growth *in utero* with an expected fetal weight lower than the 10th percentile estimated for gestational age and gender, and can be associated with abnormal Dopplers in fetal and umbilical vessels.[5,6]

PE and IUGR are heterogeneous in etiology and can be attributed to maternal, fetal and/or placental factors. Maternal risk factors associated with PE include maternal diabetes, pre-existing hypertension, renal disease and obesity.[4] Some of these maternal risk factors have also been associated with IUGR.[5] IUGR is also associated with fetal risk factors such as chromosomal abnormalities, congenital anomalies and infection.[5,6] A subset of patients develop early-onset PE together with IUGR, suggesting an overlap in the etiology underlying these complications. Histopathology of placentae from PE and IUGR pregnancies shows similar microscopic placental abnormalities, supporting the concept of similar pathophysiology underlying these two disorders.[7]

[1] Awamleh Z, Gloor GB, Han VKM. Placental microRNAs in pregnancies with early onset intrauterine growth restriction and preeclampsia: Potential impact on gene expression and pathophysiology. Accepted to BMC Medical Genomics March 6 2019.

Although the gestational age of the onset of signs and symptoms of either PE or IUGR is based on clinical outcomes and there is some overlap, it is now recognized that the early- and late- onset forms of the diseases may have different pathophysiology.[8] Early-onset forms of these complications are more severe, where the mother and infant are at higher risk of short- and long-term adverse health outcomes.[6,9] In particular, the newborns are usually born prematurely and are impacted by the morbidity of preterm birth.

Placental micro(mi)RNAs have been investigated for their role in the growth and function of the placenta, and for their potential use as diagnostic biomarkers due to their ability to enter the maternal circulation and are detectable in maternal plasma.[10] MiRNAs are a class of non-coding RNAs that are tissue-specific and are encoded in the human genome.[11,12] MiRNAs anneal to the 3`UTR of target mRNAs via sequence complementarity, and either transiently block mRNA translation or degrade the mRNA, this classifies miRNAs as epigenetic regulators.[11,12] The final product of miRNA biogenesis is an 18-22 base pair, single nucleotide strand, transcribed in the nucleus and then transported into the cytoplasm.[11,12] Placenta-specific clusters have been identified on chromosomes 14 and 19.[13,14] Differential expression analysis of miRNAs in the placenta revealed changes in miRNA expression in placentae from pregnancies complicated with PE and IUGR.[15,16] The most consistent finding is the increased expression of hypoxia-inducible miR-210 in PE placentae.[15] However, there are differences in findings among previous studies that can be attributed to: (i) the definition of patient groups; (ii) differences in the platforms used to measure expression; and (iii) analytical methods used to identify differentially expressed miRNAs.

The objective of this study was to utilize next-generation sequencing (NGS) to identify miRNAs and genes (mRNAs) expressed in the same placental tissues from three patient groups with early-onset placental disease. The patient groups include: PE only, IUGR only, PE + IUGR, and gestational-age matched controls without PE or IUGR. This analysis aims to identify differentially expressed miRNAs and genes in each disease group compared to the control group, and to integrate the two expression datasets to identify potential gene targets regulated by miRNAs.

2.2 Materials and Methods

2.2.1 Patient Recruitment

Preeclampsia was defined as hypertension (blood pressure > 140/90 mm Hg) and proteinuria (≥ 300 mg in 24 hours).[3,4] Severe PE is often associated with HELLP syndrome characterized by the onset of: edema, headache, elevated liver enzymes, and low platelet count. Patients diagnosed with PE and HELLP are indicated in Table 2.1. Intrauterine growth restriction was defined as estimated fetal weight below the 10th percentile for gestational age and gender, associated with abnormal umbilical and uterine artery Dopplers.[5,6] Patients with PE + IUGR presented with criteria aforementioned for both diseases. Only patients diagnosed prior to 34 weeks (early-onset) were included in this study. Patients with preterm labor and no other pregnancy complications before 34 weeks of gestation were recruited as controls. Women with diabetes, gestational diabetes, pre-existing hypertension, obvious chorioamnionitis (status confirmed at delivery), alcohol/drug use, chromosomal or genetic abnormalities, congenital anomalies, or

infection were excluded. All women enrolled in this study gave written informed consent for the collection of samples and information. This research was approved by the office of Human Research Ethics at Western University (REB # 102621).

2.2.2 Placental Tissue Sampling

Samples were collected from two central and two peripheral portions of the placenta within 30 minutes of delivery. Central samples were collected within a 5 cm radius from the umbilical cord insertion site and the peripheral samples were collected 2-3 cm from the edge of the placenta. Full depth 1 cm x 1 cm tissue samples were excised, and the maternal decidua was separated from the chorionic villi using gross dissection. In this study, the maternal and fetal components were separated and only the fetal components (chorionic villi) were used for analysis. The tissue samples were flash frozen in liquid nitrogen and stored at -80°C until further analysis.

2.2.3 RNA Isolation and Sequencing

Total RNA was isolated from 80-100 mg of tissue samples from each of the four regions of each placenta using the mirVana RNA isolation kit (Life Technologies, Waltham, MA, USA). Sample quantity and quality was checked using the Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Total RNA isolated from central and peripheral samples of each placenta was pooled in equal quantities for one representative total RNA sample from each patient. Samples were then sent to the Génome Québec Innovation Centre at McGill University (Montreal, QC, Canada) for library preparation and sequencing. The Illumina Truseq RNA and smRNA library preparation kits were

used to prepare mRNA and miRNA libraries respectively (Illumina, San Diego, CA, USA). Samples were sequenced on the Illumina HiSeq 2000. Complete sample journey is depicted in Supplementary Information: Figure 2.7.

2.2.4 Differential Expression Analysis

Raw read files were received from Génome Québec, FastQC was used to assess read quality. For miRNA data, sequences were aligned to miRBase version 21 index and for mRNA data, sequences were aligned to the transcriptome (GRCh37/hg19), both aligned using Bowtie2. In R v3.3.1,[17] three Bioconductor packages were used for differential expression analysis, DESeq2[18] (Benjamini-Hochberg adjusted p-value < 0.01), edgeR[19] (Benjamini-Hochberg adjusted p-value < 0.01) and ALDEx2[20] (effect > 0.8). Samples that contributed at least the median plus twice the inter-quantile range (IQR) of variance to the group were considered outliers and were subsequently removed from the analysis.[21] Only miRNAs and genes identified by at least two programs were considered significant. To adjust for covariates (fetal sex, maternal BMI, gestational age, chorioamnionitis, and mode of delivery (labor/no labor, C-section/vaginal)), surrogate variable analysis (SVA R version 3.6)[22] was used with DESeq2 and edgeR. The miRNA and gene expression data are available from the Gene Expression Omnibus (GEO) database under accession numbers GSE114349 and GSE114691, respectively.

2.2.5 Validation of miRNAs using qRT-PCR

Total RNA was reverse transcribed using TaqMan advanced miRNA cDNA synthesis kit (Life Technologies, Waltham, MA, USA). Quantification of miRNAs was completed

using the TaqMan fast advanced PCR master mix in conjunction with TaqMan miRNA expression assays (Life Technologies, Waltham, MA, USA). For miRNA normalization, miR-191-5p was used as an endogenous control.[23,24] The $\Delta\Delta C_t$ method was used for fold-change analysis. Mann-Whitney-U test was used for statistical analysis.

2.2.6 Target Prediction and Gene Ontology

Spearman correlation co-efficient was used for correlation analysis between miRNA and gene expression values. Only correlation with a $r_s \leq -0.5$ and p-value ≤ 0.01 was considered significantly negatively correlated. To further refine results only genes with a fold-change ≤ -1.5 were included for target prediction analysis. Two target prediction software were used: TargetScan (v7.1)[25] and DIANA-microT-CDS (v5.0)[26]. Criteria used for final selection of miRNA gene targets are in Supplementary Information: Table 2.2. Gene ontology analysis was completed using WebGestalt (2013)[27].

2.3 Results

2.3.1 Patient selection criteria and principal component analyses

Our aim was to select patients with primarily placental factors underlying the pregnancy complication, therefore stringent inclusion and exclusion criteria were used in the patient recruitment into the study. Patients with known maternal and/or fetal risk factors were not included (see methods). Clinical characteristics of the patient cohorts are described in Table 2.1.

Preliminary exploratory analysis of the miRNA and gene expression datasets had two aims: identification of outliers and principal component analysis (PCA) to determine if the samples clustered by disease. Identified outliers were removed prior to PCA. Based on miRNA expression data sets, one patient each was removed from the PE only group (N=19) and from the control group (N=20). Two patients were removed from the IUGR group (N=16), and none were removed from the PE + IUGR group (N=20). Based on the gene expression data set, one patient was removed from the PE only group (N=19), three patients from the IUGR only group (N=15), and four patients from the PE + IUGR group (N=16). None were removed from the control group (N=21).

Principal component analysis was then used to determine how patient samples cluster based on the expression data. Based on expression of all known human miRNAs, samples clustered separately based on disease status on the first principal component (Figure 2.1 a-c). Based on the transcriptome, samples also clustered separately based on disease status on the first principal component (Figure 2.1 d-f). Patients with PE and IUGR clustered away from controls with 15 - 40% of the variance in the data explained by the first principal component.

Table 2.1 Clinical characteristics of patients with complicated pregnancies and gestational age-matched controls.

Characteristic (Mean ± SD)	PE N=20	IUGR N=18	PE + IUGR N=20	Control N=21
Maternal Age (years)	28.6 ± 7.0	31.3 ± 5.6	32.6 ± 5.7	28.2 ± 5.0
BMI (kg/m^2)	28.9 ± 7.4	24.5 ± 4.6	28.7 ± 5.3	28.6 ± 7.5
GA at Delivery (weeks)	29.6 ± 3.1	32.3 ± 3.4	29.4 ± 2.5	30.6 ± 2.6
Sex (Females)	10 (50%)	8 (44%)	10 (50%)	11 (52%)
Mode of Delivery: C-Section (%)	15 (75%)	13 (72%)	19 (95%)	4 (19%)
C-Section with Labor (%)	6 (40%)	5 (38%)	5 (26%)	4 (100%)
Birth Weight (grams)	1300 ± 499.7[1]	1256 ± 625.6[2]	933.9 ± 342.2[3]	1803 ± 623.5
Placental Weight (grams)	342.9 ± 135.4[2]	301.5 ± 97.1[3]	244.7 ± 75.2[3,4]	462.7 ± 136.3
Birth Weight Percentile	30.4 ± 19.2	3.7 ± 2.1	4.8 ± 2.0	63.4 ± 26.5
Systolic BP (mm Hg)	173.5 ± 19.1[3,5]	118.6 ± 12.8	170 ± 14.6[3,5]	116.5 ± 15.8
Diastolic BP (mm Hg)	108.0 ± 10.3[3,5]	77.2 ± 12.6	104.3 ± 8.6[3,5]	69.24 ± 13.1
HELLP Syndrome	6 (30%)	NA	8 (40%)	NA

1) p-value < 0.05 vs. control 2) p-value < 0.001 vs. control 3) p-value <0.0001 vs. control 4) p-value <0.01 vs. PE only 5) p-value < 0.0001 vs. IUGR only

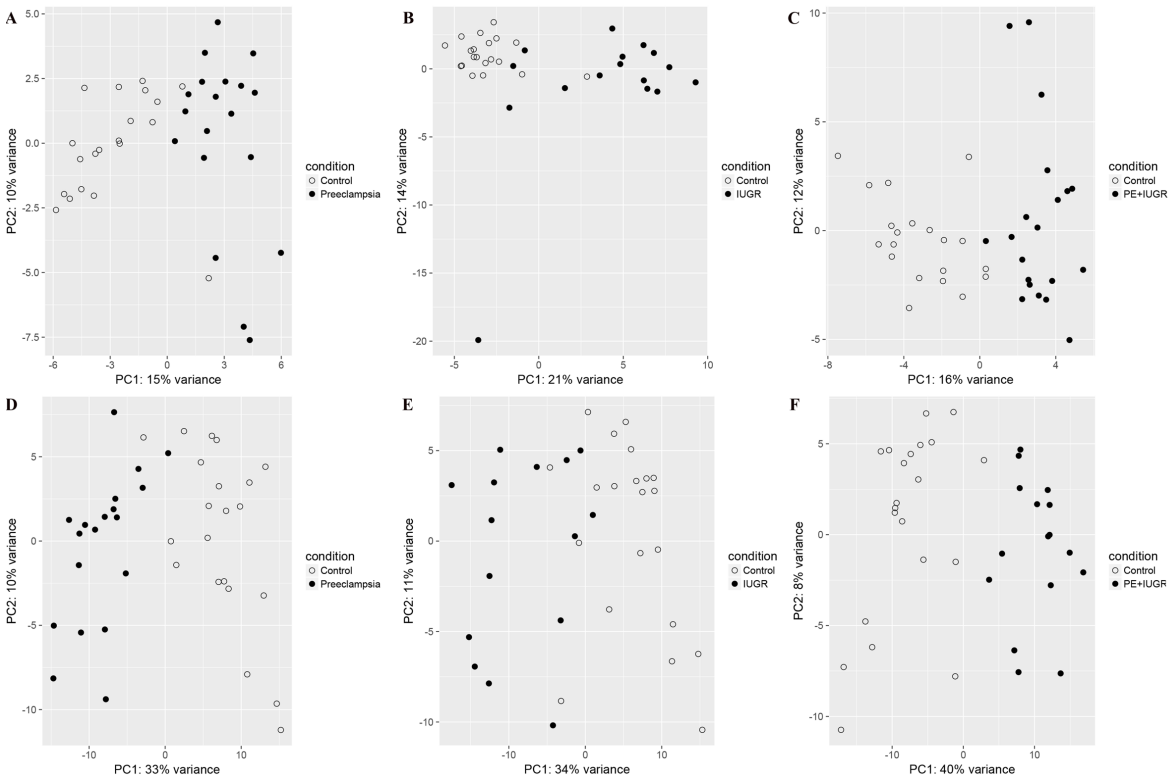


Figure 2.1 Principal Component Analysis (PCA) Plots.

PCA demonstrates clustering of patient samples based on miRNA expression (a-c) or gene expression (d-f). Gestational age-matched controls are indicated in open circles and diseased groups are indicated in closed circles. **A, D.** Preeclampsia **B, E.** Intrauterine growth restriction **C, F.** Preeclampsia and intrauterine growth restriction.

2.3.2 Differential expression analysis of placental microRNAs

To identify differentially expressed miRNAs in each patient group compared to gestational-age matched controls, three different programs namely DESeq2, edgeR, and ALDEx2 were used. Only miRNAs identified by at least two were considered significant. Using DESeq2 and edgeR, we accounted for covariates in the model such as fetal sex, maternal BMI, gestational age, and mode of delivery. We identified 11 up-regulated miRNAs in the PE only samples (Figure 2.2a), 25 upregulated and 12 downregulated miRNAs in IUGR only samples (Figure 2.2b), and 9 upregulated miRNAs in PE + IUGR samples (Figure 2.2c) (Supplementary Information: Table 2.3). Comparison of all differentially expressed miRNAs revealed 6 miRNAs that are common to all three patient groups (Figure 2.3a). All of the differentially expressed miRNAs were confirmed using qRT-PCR, with the exception of miR-520a-3p in the IUGR group (Figure 2.3b). Similarly, 3 miRNAs that were common to patients with PE only (Figure 2.3a) were also validated using qRT-PCR (Figure 2.3c).

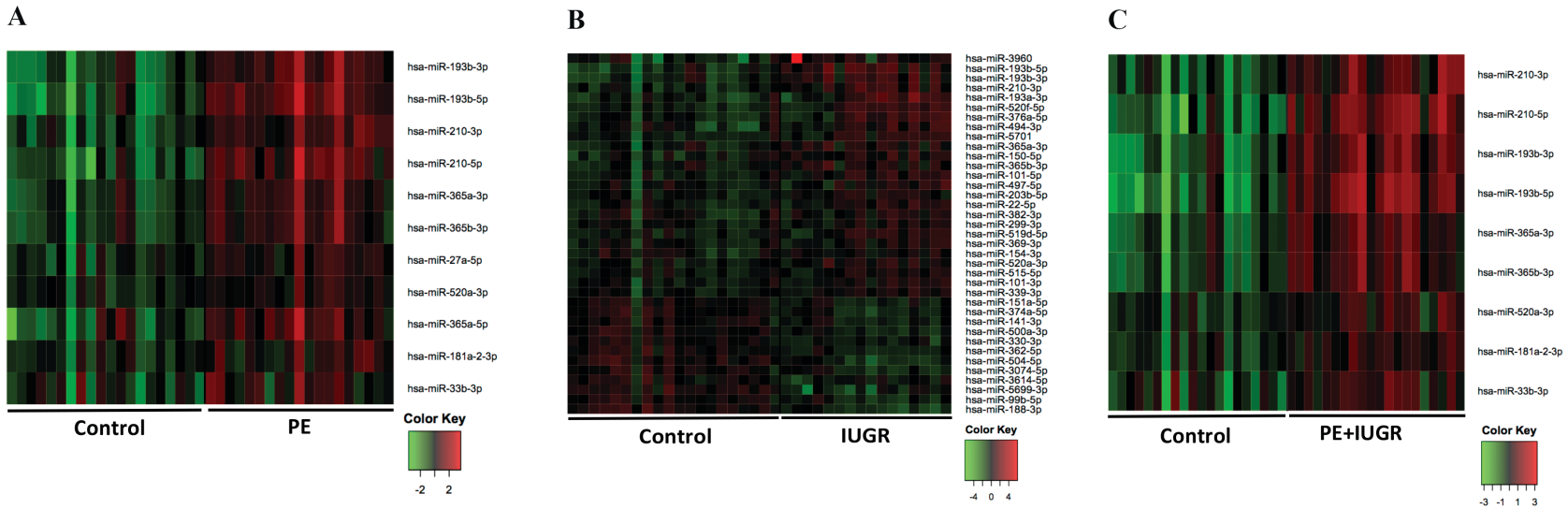


Figure 2.2 Heat maps depicting differentially expressed microRNAs in each patient group

Differential expression analysis revealed 11 upregulated miRNAs in **A.** Preeclamptic placenta, 25 upregulated and 12 downregulated miRNAs in **B.** Intrauterine growth restricted samples, and 9 upregulated miRNAs in **C.** Preeclamptic and Intrauterine growth restricted samples. Three programs were used for differential expression analysis, and only miRNAs identified by two or more programs were included in the final results.

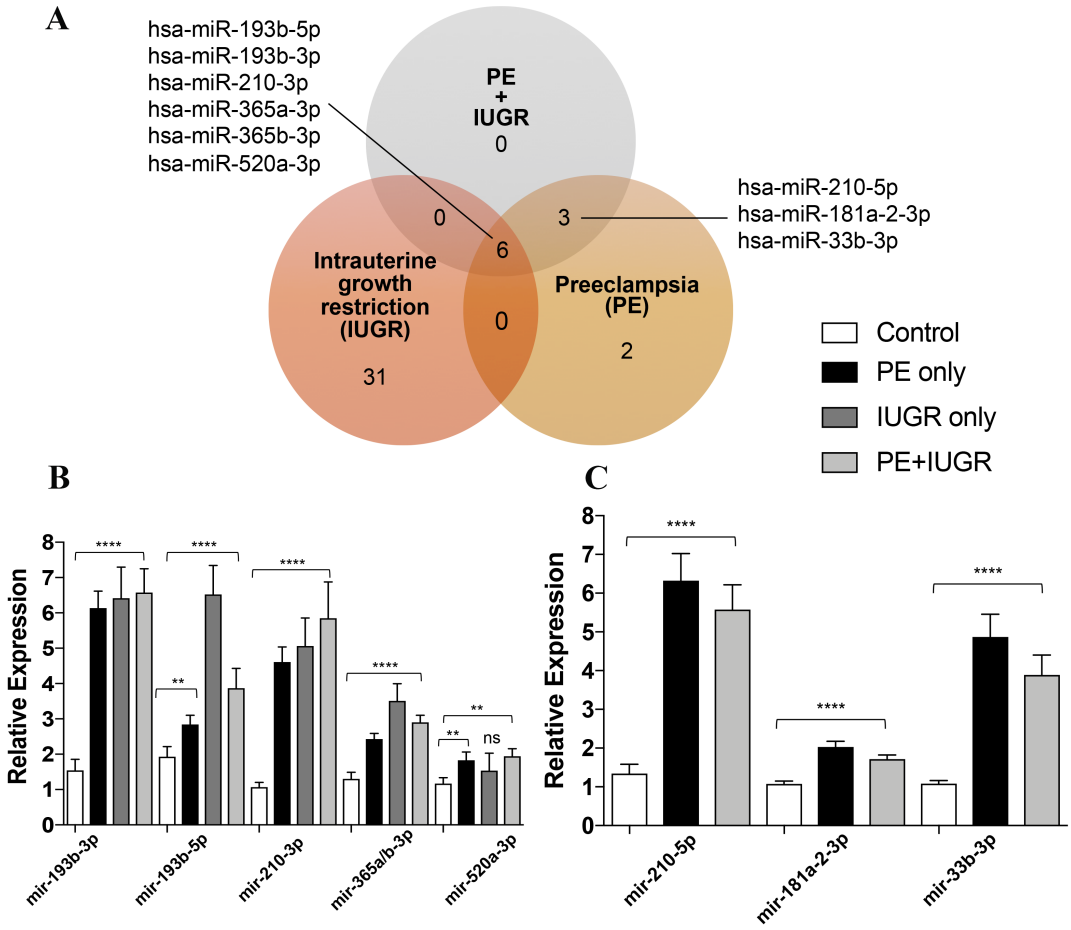


Figure 2.3 Comparing differentially expressed microRNAs between patient groups.

A. Six miRNAs are found to be common to all three patient groups, whereas three miRNAs are specific to patients with PE. **B.** Validation of miRNAs common to all three patient groups (with the exception of miR-520a-3p in the IUGR only group), and **C.** miRNAs common to PE patients using qRT-PCR. To find relative expression, the $\Delta\Delta C_T$ method was used and values were normalized to hsa-miR-191-5p expression. Data is shown as the mean ± SEM, significant differences were determined by a Mann-Whitney U test. **p-value< 0.01

2.3.3 Differential gene expression analysis in the same patient groups

The same differential expression analysis methods used for the miRNA dataset were applied to the gene expression dataset. In total, there were 275 differentially expressed genes in the PE only samples, 155 differentially expressed genes in the IUGR only samples, and 556 differentially expressed genes in PE + IUGR samples (Supplementary Information: Table 2.4). Top ten up- and down- regulated genes in each disease group are shown in Figure 2.4 (a-c). Comparison of differentially expressed genes in all patient groups revealed 22 differentially expressed genes in placental samples from all three disease groups (Figure 2.5). Other genes showed specificity to patients with PE or patients with IUGR, with 141 and 21 differentially expressed respectively. Lists of common genes between patient groups are found in Supplementary Information: Table 2.5.

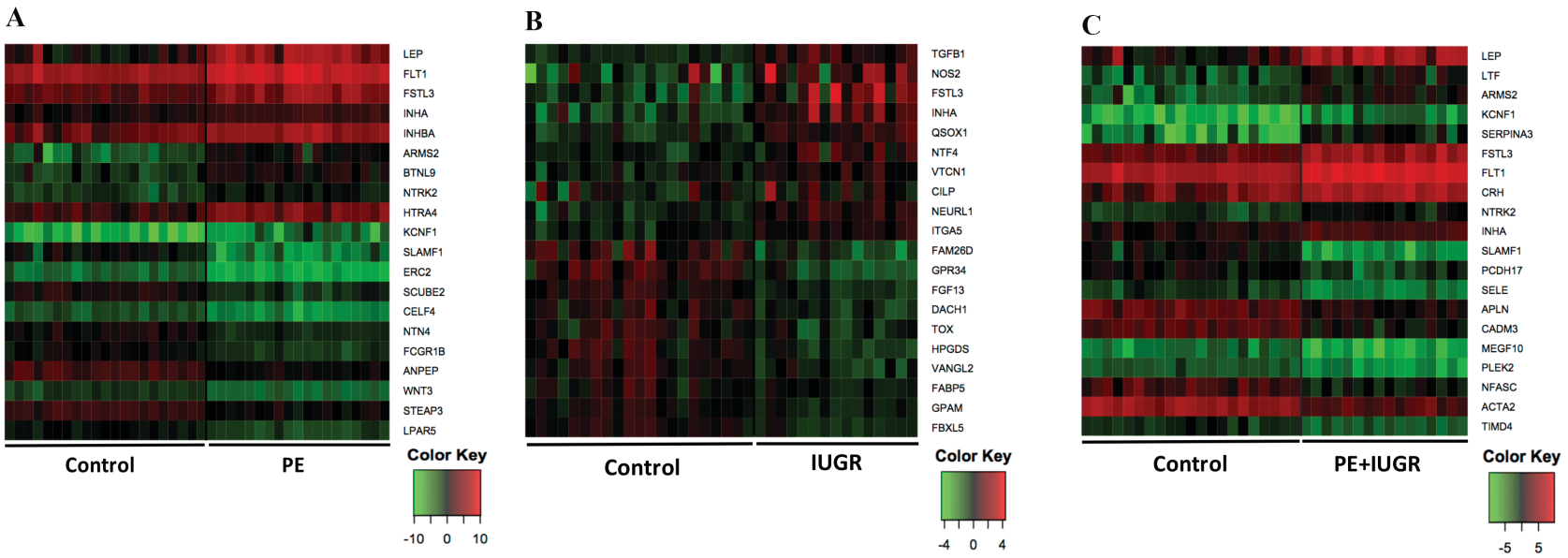
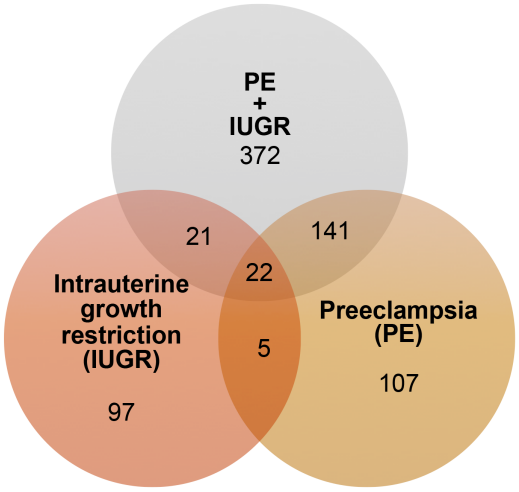


Figure 2.4 Heat maps depicting top differentially expressed genes in each patient group

Top ten upregulated and ten downregulated genes after differential expression analysis revealed **A.** 275 genes in PE only placenta, **B.** 155 genes in IUGR only placenta, and **C.** 556 genes in PE + IUGR placenta. Three programs were used for differential expression analysis, and only genes identified by two or more programs were considered differentially expressed.

A



B

Gene Name	Direction of Expression
CST6	Up-regulated
DGKZ	Up-regulated
F13A1	Down-regulated
FAM101B	Down-regulated
FGF13	Down-regulated
FSTL3	Up-regulated
GLRX	Up-regulated
HTRA1	Up-regulated
IDH1	Down-regulated
ILDR2	Up-regulated
INHA	Up-regulated
ITGA5	Up-regulated
NEURL1	Up-regulated
NFATC2	Down-regulated
NPL	Down-regulated
SLC1A6	Up-regulated
SLC9A9	Down-regulated
SLCO2A1	Up-regulated
SMIM5	Up-regulated
SNX18	Down-regulated
TGFB1	Up-regulated
VWCE	Up-regulated

Figure 2.5 Comparing differentially expressed genes between patient groups

A. Venn diagram shows the number of differentially expressed genes in each patient group compared to the control group. Three programs were used for differential expression analysis (DESeq2, edgeR, ALDEx2), and only genes identified by two or more programs were considered differentially expressed. **B**. This analysis revealed 22 genes common to all three patient groups.

2.3.4 Target Prediction and gene ontology analyses

As epigenetic regulators abundantly expressed miRNAs can impact gene expression by targeting mRNAs in the cytoplasm. Inverse correlation analysis using Spearman's correlation co-efficient identified genes that were negatively correlated in expression to differentially expressed miRNAs. Some identified genes had a significant negative correlation with one or more miRNA. To further refine the results, only candidate genes with fold-change (≤ -1.5 or ≥ 1.5) were selected for further analysis. Target prediction software programs (DIANA-microT-CDS and TargetScan) were used to identify predicted gene targets for miRNAs. A summary of criteria used to select appropriate gene targets is found in Supplementary Information: Table 2.2. This analysis resulted in 16 candidate gene targets (Figure 2.6a) (Supplementary Information: Table 2.6). The majority of candidate gene targets identified were found in PE only or PE + IUGR groups. Interestingly, there were candidate genes such as *APLN*, *CSF1*, *FZD5*, and *TYRO3* that are potential targets of multiple miRNAs (Figure 2.6a). Gene ontology analysis of the 16 candidate gene targets revealed that most genes are involved in important cellular functions including proliferation, migration, communication and adhesion (Figure 2.6b).

A

Candidate Gene Target	Patient Group	Targeting miRNA
APLN	PE + IUGR	miR-193b-5p, miR-193b-3p, miR-210-5p
C3AR1	PE, PE + IUGR	miR-210-5p
CCR1	PE	miR-193b-5p
CSF1	PE + IUGR	miR-193b-5p, miR-210-5p
FGF13	PE, IUGR, PE+IUGR	miR-193b-5p
FZD5	PE + IUGR	miR-365a-3p, miR-365b-3p
IL12RB2	PE, PE + IUGR	miR-193b-5p
ITGAM	PE + IUGR	miR-210-5p
NTN4	PE	miR-193b-5p
NRP2	PE + IUGR	miR-193b-5p
PTGS1	PE + IUGR	miR-193b-5p
SELE	PE + IUGR	miR-210-5p
TLR7	PE + IUGR	miR-193b-5p
TYRO3	PE, PE + IUGR	miR-193b-5p, miR-210-5p
VAV1	PE, PE + IUGR	miR-210-5p
WNT3	PE	miR-210-5p

B

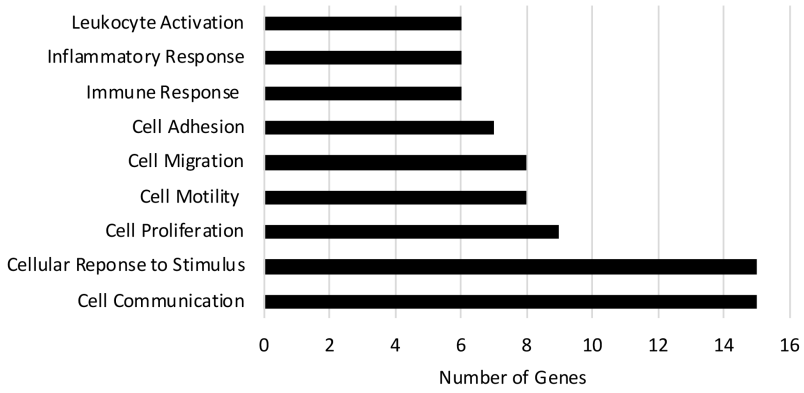


Figure 2.6 Target prediction and gene ontology analyses.

A. Identified candidate gene targets for miRNAs that have been validated with qRT-PCR. Spearman correlation was used to identify genes that inversely correlate in expression to validated miRNAs, in each patient group. Candidate gene targets were identified using prediction software DIANA-microT-CDS and TargetScan. **B.** Gene ontology analysis for identified candidate genes.

2.4 Discussion

Our study identified dysregulated miRNAs and genes in placental samples that are common to three patient groups with early-onset pregnancy complications. More specifically, 6 miRNAs and 22 genes were found to be commonly differentially expressed in patients with early-onset PE, IUGR, and PE + IUGR, compared to gestational age matched controls. However, some miRNAs and genes remained disease specific. To integrate the miRNA and gene expression datasets, inverse correlation analysis revealed candidate gene targets primarily involved in cell functions such as proliferation and migration.

MicroRNAs identified to be common to all patient groups, are some of the most commonly identified miRNAs in the literature. MicroRNAs identified in this study have been identified in studies measuring miRNA expression in placental samples from primarily PE pregnancies. Studies assessing global miRNA expression in placenta from IUGR pregnancies are scarce, potentially due to heterogeneity of etiologies underlying this condition. Studies utilizing global miRNA expression analyses in PE placenta with overlapping findings to this present study are found in Supplementary Information: Table 2.7. One of the most well characterized miRNAs in pregnancy complications is miR-210.[15,28] Under hypoxic conditions, miR-210 is upregulated by the transcription factor HIF-1α.[28] The intrauterine environment in pregnancies complicated by PE and/or IUGR has been suggested to be hypoxic due to decreased perfusion of maternal blood to the feto-placental unit. In our study, we identified that miR-210-3p is common to all patient groups, whereas miR-210-5p is common to patients with PE (Figure 2.3). Gene targets of

miR-210 have also been investigated to identify the impact of the upregulation of miR-210 on the placenta during disease.[28] Identified targets for miR-210 include *EFNA3* and *HOXA9*, which are important for cell functions such as migration and invasion.[28,29] In our study, downregulated predicted targets of miR-210-5p are also involved in cell migration, they include *APLN*, *CSF1*, *ITGAM*, *C3AR1*, and *SELE* (Figure 2.6, Supplementary Information: Table 2.6). These novel gene targets for miR-210-5p have been identified to be downregulated in patients with preeclampsia (± IUGR) in our study (Supplementary Information: Table 2.4). Gene targets such as *APLN* and *C3AR1* have been shown in the literature to be downregulated in placental samples from PE patients with an unknown underlying mechanism for dysregulation. [30,31]

Other miRNAs such as miR-193b-3p/5p, and miR-365a/b-3p have also been previously identified in studies measuring miRNA expression in placental samples from PE pregnancies[32-34] (Table 2.7). Identified gene targets for miR-193b-3p/5p are enriched in gene ontology categories important for cell functions such as cell proliferation, adhesion, and migration (Figure 2.6a). Zhou et al. (2016) showed increased expression of miR-193b-3p and -5p in PE patients, and that miR-193b-3p was found to decrease migration and invasion of HTR-8/SVneo cells.[34] We also identified *FZD5* (frizzled 5) as a candidate gene target for miR-365a/b-3p, which belongs to the frizzled family of proteins, known to be important during placental development (Figure 2.6). Frizzled 5 mRNA and protein have also been reported to be down regulated in PE placenta.[35] In the IUGR only group the majority of miRNAs remained unique to that patient group (31/37). Only 2 miRNAs overlap with findings in the literature, however, in the opposite direction

of expression. In our study miR-515-5p and miR-519d-5p were upregulated, and in the study by Higashijima et al. that utilized NGS these two miRNAs are downregulated.[16] Interestingly, of the 37 differentially expressed miRNAs in the IUGR only group, 4 belonged to the C19MC (520a-3p, 520f-5p, 515-5p, 519-5p) and 6 belonged to the C14MC (299-3p, 494-3p, 376a-5p, 382-3p, 154-3p, 369-3p), the two miRNA clusters known to be placenta-specific.[1]

Differential gene expression analysis in placentae from the same patient populations, was crucial in identifying potential gene targets for the validated miRNAs. In addition, comparison of all patient groups revealed a subset of genes common to the three diseased patient groups. A number of the differentially expressed genes the we have identified in our diseased patient cohorts, have previously been identified to be dysregulated in placental samples from complicated pregnancies. One example includes *INHA* (inhibin) and *FSTL3* (follistatin-like 3), both of which have been reported to be upregulated in PE placenta.[36,37] In this study *INHA* and *FSTL3* are upregulated in pregnancies with IUGR as well (Figure 2.4, Supplementary Information: Table 2.4). Differences observed between this current study and other studies assessing global expression can be attributed to (i) strict patient selection criteria, (ii) platform used to measure miRNA and gene expression, and (iii) methods used for differential expression analysis.

Patients in this study strictly had early-onset disease, and by excluding a number of maternal and fetal predisposing factors, the patients were better standardized as to the pregnancy complications.[8] Despite this current study being the largest to integrate miRNA and gene expression data in these patient cohorts to our knowledge, a larger

sample number or additional replication groups would be beneficial. However, strict patient selection criteria were beneficial, and this is demonstrated in principal component analyses based on miRNA and gene expression data in these patient groups that showed clear segregation by disease status on the first principal component (Figure 2.1). In addition, previous studies on PE do not always stratify patients into PE only and PE + IUGR, whereas in our study, these two patient populations were segregated and treated as distinct populations. Recent studies have shown distinct gene expression profiles for different subclasses of PE (late vs. early, ± IUGR).[38,39] Leavey et al. combined microarray gene expression data from previously published studies in PE placentae, and conducted unsupervised clustering analysis.[39] The study identified three main subclasses of PE based on gene expression - late-onset PE, which is mostly associated with term deliveries and maternal risk factors ("maternal PE"); early-onset PE, which is likely placental in origin and is more frequently associated with IUGR ("canonical PE"); and a third subclass of PE that is also severe and can co-occur with IUGR but is likely due to poor maternal-fetal compatibility ("immunologic PE").[39] Interestingly, cluster analysis based on DNA methylation data in PE placentae revealed a similar clustering pattern into the three subclasses of PE.[40] In this study, 8763 and 340 differentially methylated sites were found in the "canonical" and "immunologic" subclasses respectively.[40] Wilson et al. also showed differentially methylated sites, as many as 599 sites, in EO-PE and only 5 in late-onset PE.[41] These studies show differences in gene expression and in epigenetic mechanisms between subclasses of PE, emphasizing that each subclass has a unique underlying pathophysiology. In addition, these studies demonstrate the benefits of

combining epigenetic and gene expression data to improve our understanding of molecular mechanisms in the placenta.

Next-generation sequencing used in this study to assess miRNA and gene expression has many advantages compared to microarray platforms.[42,43] The detection capacity of microarrays is limited by the pre-determined probes on the array platforms, whereas NGS data can be used to align to the most updated sequence information, which is beneficial for the discovery of new miRNAs and genes. Moreover, NGS has a wider dynamic range and is therefore capable of detecting miRNAs and mRNAs that are expressed at low levels. Interestingly, from our miRNA study, we identified that our findings overlap more with studies that have also used sequencing techniques compared to studies utilizing microarray technology.[34,44]

Lastly, the stringency of statistical analyses to identify differentially expressed miRNAs and genes is beneficial to increase confidence but could result in the exclusion of some miRNAs and genes that may contribute to pathogenesis of PE and/or IUGR. On the other hand, methods for analyzing high-throughput data are currently not standardized, particularly normalization and identification of differences.[45] Each program in this study utilized a different approach to the normalization and differential expression analysis. Therefore, using stringent cut-off values and including only miRNAs and genes identified by two or more programs increased the reliability of our findings compared to using a single approach for differential expression.

2.5 Conclusion

In summary, this study shows that maldevelopment of the placenta early in gestation may manifest itself into different complex diseases in the second and third trimester, however some common underlying pathophysiological mechanisms remain in the placenta. Identification of common miRNAs and genes that are dysregulated in all three patient groups supports this observation. The gene expression data set allowed us to identify potential novel gene targets that are primarily involved in cellular processes important for placental growth and function. Identified candidate gene targets can be further experimentally validated to demonstrate miRNA-mRNA interactions and to assess the impact of miRNAs on gene expression. Identified miRNAs can also by assessed for their efficacy as diagnostic biomarkers for these diseases. Continued integration of epigenetic and gene expression data in larger disease cohort can validate findings and expand our understating of molecular mechanisms impacting placental growth and function.

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2.7 Supplementary Information

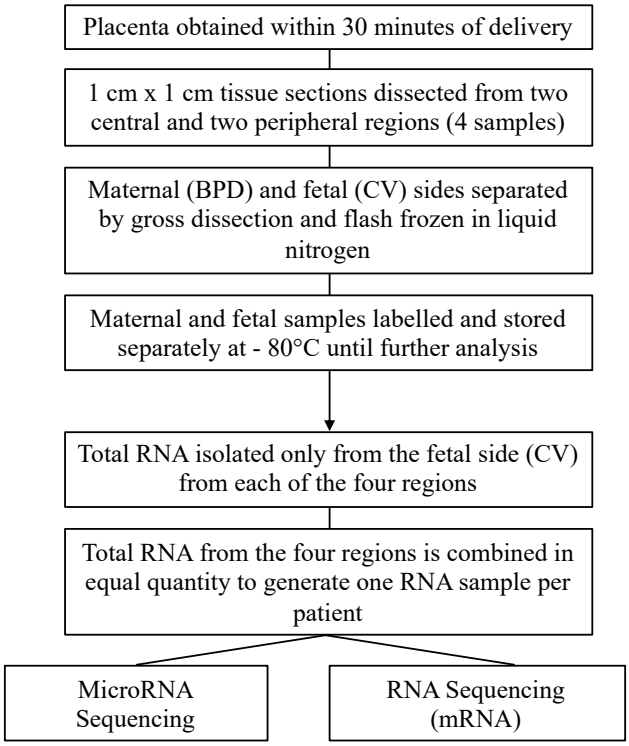

Figure 2.7 Journey of placental samples from delivery room to sequencing facility.

Table 2.2 Criteria for selection of appropriate gene targets for microRNAs.

Two categories of criteria were used to identify potential gene targets for microRNAs in each disease group. All microRNAs included in the final analysis meet all category 1 criteria and meet at least one of the category 2 criteria.

Category 1 Criteria (Must meet all)
Spearman correlation co-efficient between miRNA and gene is ≤ -0.5 (between upregulated miRNAs and downregulated genes OR downregulated miRNAs and upregulated genes)
Significant correlation BH adjusted p-value ≤ 0.01
Gene relative fold change at minimum 1.5-fold lower or higher than expression in control samples
Gene is predicted target by at least one target prediction program (TargetScan, DIANA-microT-CDS)
Category 2 Criteria (Meet one or more)
Gene target predicted by multiple prediction tools (i.e. DIANA-microT-CDS and TargetScan)
Gene is predicted target for multiple microRNAs
Gene target is found in more than 1 disease group
Published literature supporting link to pregnancy or pregnancy complication
Pathway enrichment supports link to pregnancy or pregnancy complications

Table 2.3 List of differentially expressed miRNAs in each disease group compared to gestational age-matched controls (* BH adjusted p-value, + Effect Size).

	MicroRNA	DESeq2*	edgeR*	ALDEx2+	Direction of effect
PE	hsa-miR-193b-5p	9.1E-15	7.6E-09	2.175	Up
	hsa-miR-193b-3p	1.8E-10	1.3E-06	1.988	Up
	hsa-miR-210-5p	3.4E-11	1.3E-06	2.091	Up
	hsa-miR-210-3p	4.8E-10	1.3E-06	2.018	Up
	hsa-miR-365a-3p	1.6E-07	1.1E-05		Up
	hsa-miR-365b-3p	1.1E-06	5.4E-05		Up
	hsa-miR-181a-2-3p	7.1E-06	6.6E-04		Up
	hsa-miR-365a-5p	1.3E-05	2.2E-03		Up
	hsa-miR-27a-5p	7.4E-05	2.2E-03		Up
	hsa-miR-33b-3p	8.0E-04	4.2E-03		Up
	hsa-miR-520a-3p	3.8E-04	1.3E-05		Up
IUGR	hsa-miR-210-3p	3.7E-07	1.3E-04	1.344	Up
	hsa-miR-193b-3p	2.8E-07	1.1E-04	1.151	Up
	hsa-miR-193b-5p	2.8E-07	6.8E-04	1.262	Up
	hsa-miR-339-3p	4.4E-04		1.026	Up
	hsa-miR-520a-3p	2.7E-03		0.880	Up
	hsa-miR-520f-5p	1.1E-07	4.4E-05		Up
	hsa-miR-188-3p	1.1E-07	4.4E-05		Down
	hsa-miR-3614-5p	8.8E-10	5.6E-05		Down
	hsa-miR-99b-5p	4.3E-07	7.2E-05		Down
	hsa-miR-376a-5p	1.0E-06	1.1E-04		Up
	hsa-miR-365a-3p	2.5E-05	3.5E-04		Up
	hsa-miR-5701	1.0E-05	4.8E-04		Up
	hsa-miR-494-3p	7.8E-05	9.9E-04		Up
	hsa-miR-3074-5p	1.2E-05	9.9E-04		Down
	hsa-miR-299-3p	7.4E-05	1.1E-03		Up
	hsa-miR-365b-3p	1.0E-04	1.4E-03		Up
	hsa-miR-369-3p	1.6E-04	2.2E-03		Up
	hsa-miR-151a-5p	8.1E-06	2.6E-03		Down
	hsa-miR-193a-3p	8.6E-06	2.7E-03		Up
	hsa-miR-500a-3p	1.2E-05	2.7E-03		Down
	hsa-miR-382-3p	7.5E-04	2.7E-03		Up
	hsa-miR-362-5p	3.2E-05	2.7E-03		Down
	hsa-miR-330-3p	2.1E-05	2.8E-03		Down
	hsa-miR-141-3p	8.6E-05	3.7E-03		Down
	hsa-miR-22-5p	1.2E-04	3.9E-03		Up
	hsa-miR-150-5p	1.0E-04	3.9E-03		Up
	hsa-miR-374a-5p	4.1E-05	3.9E-03		Down
	hsa-miR-101-5p	4.3E-05	4.4E-03		Up
	hsa-miR-497-5p	7.7E-04	5.9E-03		Up
	hsa-miR-3960	3.1E-07	6.7E-03		Up
	hsa-miR-504-5p	7.7E-05	6.7E-03		Down
	hsa-miR-519d-5p	2.8E-04	6.7E-03		Up
	hsa-miR-203b-5p	2.9E-04	7.0E-03		Up
	hsa-miR-515-5p	1.8E-04	7.0E-03		Up
	hsa-miR-154-3p	1.1E-03	7.7E-03		Up
	hsa-miR-101-3p	1.3E-04	9.2E-03		Up
	hsa-miR-5699-3p	3.9E-04	9.8E-03		Down
PE + IUGR	hsa-miR-210-3p	7.5E-13	4.4E-06	2.082	Up
	hsa-miR-210-5p	2.7E-13	5.8E-06	2.435	Up
	hsa-miR-193b-3p	2.4E-12	4.6E-06	2.644	Up
	hsa-miR-193b-5p	8.8E-13	9.0E-05	2.537	Up
	hsa-miR-365a-3p	6.6E-09	3.1E-05	2.230	Up
	hsa-miR-365b-3p	8.0E-08	2.7E-04	1.995	Up
	hsa-miR-520a-3p	3.4E-05		1.736	Up
	hsa-miR-181a-2-3p	1.1E-06	1.9E-04	1.914	Up
	hsa-miR-33b-3p	2.5E-05	7.6E-03		Up

Table 2.4 List of differentially expressed genes in each disease group compared to gestational age-matched controls (* BH adjusted p-value, + Effect Size).

	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect
PE	*ABCA7*	1.7E-08	5.6E-04		Up	*CD180*	2.5E-07	5.3E-03		Down
	AC110619.2	8.3E-04		1.562	Up	CD200R1	9.95E-05		-1.536	Down
	ACKR2	1.1E-05	4.6E-03		Up	*CD5L*	6.4E-13	1.7E-03		Down
	ADAM12	9.7E-06		1.490	Up	*CD63*	7.2E-03		1.603	Up
	ADAMTS7	4.7E-07	6.5E-03		Down	*CEBPB*	1.5E-03		1.464	Up
	ADH5	1.3E-04	5.9E-03		Down	*CELF4*	2.4E-06	2.0E-03		Down
	ALPK3	1.8E-07	5.6E-04	1.565	Up	*CELSR1*	1.5E-08	1.7E-03		Up
	AMACR	7.2E-04	7.1E-03		Down	*CFI*	1.1E-05	4.6E-03		Down
	ANKRD24	1.3E-05		1.461	Up	*CLDN6*	9.0E-09	6.5E-03		Up
	ANO1	1.4E-04		1.437	Up	*CLDN9*	3.2E-09	7.1E-03	1.376	Up
	ANPEP	4.3E-06	5.7E-03		Down	*CLEC11A*	7.5E-04	9.3E-03		Down
	AP000350.10	6.2E-06	8.3E-03	1.569	Up	*CLIC3*	2.2E-03		1.281	Up
	ARHGAP18	2.4E-04	9.5E-03		Down	*CORO1A*	3.5E-07	2.1E-04		Down
	ARHGEF4	3.0E-15	6.3E-05	2.160	Up	*CORO2A*	8.0E-09		1.308	Up
	ARMS2	1.5E-21	2.1E-04	2.126	Up	*CPNE5*	2.2E-05	4.1E-04		Up
	ASTE1	1.9E-06	3.0E-03	1.690	Up	*CRH*	7.0E-25	1.2E-03	1.663	Up
	B4GALNT2	3.2E-14	7.4E-03		Up	*CST6*	1.8E-19	4.4E-05	1.807	Up
	BCAR3	3.5E-06	6.6E-03		Up	*CTD-2014B16.3*	5.9E-06	5.6E-04		Up
	BCL6	5.0E-14	5.5E-05	2.248	Up	*CTSC*	6.3E-05	6.4E-03	-1.851	Down
	BCORL1	4.0E-05		1.345	Up	*CXCL2*	2.3E-05	1.2E-03		Up
	BHLHE41	1.5E-08	9.7E-05		Down	*CXorf57*	2.7E-04	7.4E-03		Down
	BLVRA	1.4E-04	6.2E-03		Down	CYBB	5.00E-05		-1.418	Down
	BMX	3.5E-08	5.2E-03	-1.595	Down	*CYP26A1*	1.4E-11	5.5E-03		Up
	BTNL9	2.6E-17	1.5E-03	2.085	Up	*DAB2IP*	9.7E-05		1.609	Up
	C10orf90	1.5E-08	4.9E-03	1.609	Up	DDR2	3.82E-05		-1.474	Down
	C12orf75	1.3E-18	7.5E-08	2.445	Up	*DDX26B*	7.9E-05	3.1E-03		Down
	C3	8.2E-04	5.2E-03		Down	*DGKZ*	7.5E-04		1.616	Up
	C3orf35	1.6E-04	3.1E-03		Up	*DIO2*	1.8E-12	6.5E-03		Up
	CA4	5.6E-11		1.738	Up	*DLG2*	3.8E-07	4.5E-04		Down
	CALM1	3.8E-08	3.3E-03		Up	*DNAH11*	3.4E-13	2.5E-05		Up
	CARKD	1.9E-03		1.593	Up	*DNM2*	9.0E-06	4.0E-03	2.242	Up
	CASP1	6.54E-03		-1.449	Down	*DOK3*	1.1E-05	3.9E-03	-1.440	Down
	CBX4	2.3E-04	7.3E-03		Down	*DSCR4*	5.6E-11	1.9E-04	1.899	Up
	CCDC113	1.1E-07	5.3E-03	1.651	Up	*DVL1*	9.1E-04		1.566	Up
	CCDC183	4.7E-16	1.0E-06	2.211	Up	*EAF1*	1.1E-06	1.2E-03		Up
	CCR1	3.9E-04	6.2E-03		Down	*EBI3*	1.6E-09	9.1E-03	1.728	Up

	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect
PE	*EDIL3*	1.4E-03	6.2E-03		Down	*GLB1*	1.0E-04	3.6E-03		Down
	EFEMP1	2.4E-06	4.1E-04		Down	*GLRX*	1.5E-04		1.441	Up
	EFHD1	5.2E-08	2.8E-03		Up	*GOLGA6L6*	5.1E-04	1.4E-03		Up
	EFNB1	1.1E-04		1.682	Up	*GPIHBP1*	2.4E-10		1.522	Up
	EGLN3	1.3E-10	1.8E-03		Up	*GPR45*	1.2E-05	9.5E-03		Up
	EMID1	2.4E-06	1.2E-03		Down	*GSE1*	1.5E-05	6.9E-03	1.559	Up
	ENG	1.7E-12	4.0E-03	1.818	Up	*GUCA2A*	1.6E-06		1.499	Up
	ENPP2	9.7E-04	8.3E-03		Down	HADH	7.92E-03		-1.196	Down
	ERC2	1.4E-07	6.4E-03		Down	HAVCR2	1.49E-03		-1.461	Down
	ERGIC1	1.6E-05	6.8E-03	1.803	Up	*HCAR3*	2.6E-06	6.6E-03		Up
	ERO1L	2.5E-07	6.2E-03		Up	*HCK*	9.4E-05	6.5E-03		Down
	ERRFI1	1.4E-07	7.1E-03		Up	*HEXB*	9.7E-09	8.3E-03	1.582	Up
	EVL	7.9E-05	1.7E-03		Down	*HMHA1*	3.0E-13	9.9E-04	1.933	Up
	EZR	1.2E-05		1.707	Up	HNMT	5.02E-04		-1.698	Down
	F13A1	4.1E-08	1.1E-03	-1.813	Down	*HS6ST2*	5.5E-06	2.8E-03	-1.785	Down
	FAM101B	9.8E-07	8.9E-04	-1.633	Down	*HTRA1*	4.5E-21	8.5E-06	2.250	Up
	FAM110B	1.6E-04	6.8E-03		Down	*HTRA4*	3.6E-28	3.1E-06	2.042	Up
	FAM120AOS	9.8E-06	5.3E-03		Up	*IDH1*	5.0E-10	3.1E-06	-1.512	Down
	FAM198A	9.0E-06		1.596	Up	*IL8*	1.2E-05	2.1E-04		Up
	FAM219A	6.3E-03		1.423	Up	*ILDR2*	1.6E-03		1.302	Up
	FAM64A	1.5E-06	7.6E-03		Down	*ILVBL*	1.8E-06		1.923	Up
	FAM83A	7.3E-05	4.9E-03		Up	*INHA*	1.3E-23	1.6E-05	2.270	Up
	FCER1G	2.35E-05		-1.627	Down	*INHBA*	4.5E-21	1.6E-03	1.863	Up
	FCGR1B	1.7E-04	4.3E-03		Down	*INPP5B*	1.4E-04	3.9E-03		Up
	FGF13	2.4E-05	6.5E-03	-1.597	Down	*IQSEC1*	9.7E-04		1.569	Up
	FKBP2	2.7E-03		1.436	Up	*IRF2BPL*	7.7E-03		1.553	Up
	FLJ00418	5.7E-09	9.5E-03		Up	*ITGA5*	2.1E-09	5.4E-04	1.617	Up
	FLNB	3.8E-07	6.6E-03		Up	*KCNF1*	1.9E-12	1.7E-03		Up
	FLT1	3.6E-28	3.1E-06	2.756	Up	*KISS1R*	3.9E-08	5.6E-04		Up
	FOLR2	1.09E-04		-1.597	Down	KLHL42	2.92E-03		-1.615	Down
	FPR1	7.58E-05		-1.768	Down	*KRT19*	8.1E-09		1.547	Up
	FSTL3	6.4E-21	6.0E-05	2.037	Up	*KRTAP26-1*	7.6E-16	6.3E-08		Up
	GALE	1.5E-03		1.444	Up	*LAMA2*	1.2E-05	6.2E-03		Down
	GAPDH	2.0E-06	5.3E-03		Up	*LAMB1*	2.3E-06	8.3E-03		Down
	GBA	1.3E-09	1.1E-04	1.733	Up	*LEP*	1.8E-22	1.0E-06	2.338	Up
	GDPD4	7.4E-05	7.1E-03		Down	*LHFP*	3.2E-04	9.6E-03		Down
	GFOD2	3.4E-05		1.745	Up	*LIMCH1*	2.1E-10	6.2E-03	1.487	Up

	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect
PE	*LIMS1*	2.7E-04	6.8E-03		Up	*PGD*	1.15E-03		-1.249	Down
	LONRF2	4.2E-09	1.4E-04		Down	*PHYHIP*	3.0E-15	6.0E-05	1.601	Up
	LPAR5	3.6E-05	7.1E-03		Down	*PIWIL4*	1.5E-06	2.9E-03	-1.536	Down
	LY6D	4.7E-16	4.0E-03	1.667	Up	*PJA1*	5.2E-05	8.7E-03	1.488	Up
	MAP7D2	1.1E-04	8.0E-03	-1.666	Down	*PLA2G16*	2.4E-09	2.3E-03	1.918	Up
	MAST4	7.4E-06	3.6E-03	1.546	Up	*PNCK*	4.0E-19	2.2E-03	1.498	Up
	MCM6	6.93E-03		-1.214	Down	*PPL*	2.1E-12	2.5E-05	1.943	Up
	MED13L	2.0E-04		1.593	Up	*PPP1R12C*	1.1E-04		1.753	Up
	METTL7A	1.7E-04	8.3E-03		Down	*PPP1R1C*	4.4E-10	2.9E-03		Up
	MFAP5	1.1E-05		1.715	Up	*PRDX6*	2.0E-04	6.6E-03		Up
	MICAL3	1.9E-06		1.536	Up	*PROCR*	5.9E-14	2.6E-04	2.001	Up
	MIDN	3.6E-04		1.592	Up	*PRSS54*	5.6E-11	6.0E-05	1.675	Up
	MIF	1.0E-05	9.6E-03		Up	PTPN9	3.99E-03		-1.530	Down
	MOCS2	9.3E-05	7.2E-03	-1.446	Down	*PVRL4*	1.9E-12	7.0E-03	1.592	Up
	MORC4	3.1E-05	2.9E-03		Down	*QPCT*	3.3E-15	2.8E-03	2.108	Up
	MPP1	6.13E-04		-1.574	Down	*RAB3IL1*	5.5E-06	2.8E-03	-1.471	Down
	MYH10	6.4E-05	6.6E-03		Down	*RASSF4*	1.2E-05	6.2E-03	-1.630	Down
	MYO7A	9.3E-05	6.8E-03		Down	RASSF6	9.67E-05		-1.637	Down
	MYO7B	2.9E-23	1.4E-06	2.269	Up	*RASSF7*	1.5E-06	8.7E-03		Up
	NCKAP1L	2.22E-04		-1.693	Down	*RCAN1*	3.2E-06	2.4E-03	-1.734	Down
	NDRG1	1.6E-12	5.6E-04	1.690	Up	*RDH13*	2.6E-10		1.509	Up
	NEK11	5.9E-14	2.3E-04	1.858	Up	*RHBDD2*	9.2E-07	1.2E-03	1.666	Up
	NEURL1	5.6E-11	4.3E-03	1.731	Up	*RP11-211G3.3*	6.8E-12	6.0E-05		Up
	NFATC2	1.02E-03		-1.569	Down	*RP11-986E7.7*	4.5E-09	1.2E-03		Up
	NFKBIZ	1.3E-04	8.1E-03		Up	*RUSC2*	2.5E-05	2.4E-03		Up
	NPL	2.77E-04		-1.416	Down	*S100A16*	8.4E-04	9.4E-03		Up
	NTN4	6.3E-07	2.0E-03		Down	*S100A6*	4.9E-05		1.582	Up
	NTRK2	2.5E-20	3.6E-05	1.896	Up	*SASH1*	2.9E-10	2.0E-03		Up
	OCRL	2.6E-05	7.5E-03		Up	*SASH3*	6.1E-06	1.9E-03	-1.680	Down
	OSR1	3.7E-05	9.3E-03		Down	*SBSPON*	6.4E-05	7.2E-03		Down
	P4HA1	2.3E-06	8.1E-03		Up	*SCARB1*	5.8E-07	6.0E-03	1.904	Up
	PAM	2.2E-04		1.447	Up	*SCN11A*	7.3E-05	5.6E-03		Down
	PAPPA2	2.7E-20	5.2E-03	1.844	Up	*SCN7A*	2.0E-06	6.2E-03		Down
	PC	2.4E-06	3.4E-03	-1.613	Down	*SCUBE2*	7.2E-08	2.6E-03		Down
	PDK2	2.5E-05	4.9E-03		Down	*SDC3*	1.3E-05	2.8E-03		Up
	PDLIM2	2.2E-04		1.545	Up	*SEL1L3*	3.7E-05	3.4E-03	-1.539	Down
	PDZD7	1.9E-12	5.8E-03	1.822	Up	*SERPINA3*	3.7E-14	3.3E-06		Up

	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect
PE	*SFXN3*	3.9E-10	4.4E-04	1.798	Up	*SIGLEC6*	4.0E-11	1.2E-03		Up
	SH3PXD2A	2.7E-14	3.2E-06	2.568	Up	*SIPA1L1*	1.0E-04		1.530	Up
	SLAMF1	1.1E-19	2.2E-03	-1.874	Down	*TRIP10*	6.0E-06	6.8E-03		Up
	SLC16A3	9.2E-07		1.704	Up	*TRPM2*	1.9E-05	3.1E-03		Down
	SLC1A6	8.5E-11	2.8E-03		Up	*TYRO3*	1.9E-05	5.3E-03		Down
	SLC27A2	1.9E-06	1.1E-03	-1.368	Down	TP53I11	4.91E-03		-1.318	Down
	SLC28A2	1.4E-06	8.7E-03		Down	*TPBG*	6.4E-16	6.1E-05		Up
	SLC35D1	1.1E-06	5.6E-04	-1.619	Down	*TPI1*	5.8E-05	9.5E-03		Up
	SLC6A6	2.9E-06		1.282	Up	*ULBP1*	7.1E-06	4.7E-05		Up
	SLC6A8	1.5E-12	1.1E-03	1.558	Up	*VAV1*	3.0E-06	2.8E-03		Down
	SLC9A9	1.2E-07	7.0E-04	-1.725	Down	*VSIG4*	4.8E-08	1.1E-03		Down
	SLCO2A1	3.5E-12	2.2E-03	1.865	Up	*VWCE*	4.6E-13	7.7E-04		Up
	SMAGP	5.8E-05	1.7E-03		Down	*WLS*	7.7E-09	9.7E-05		Down
	SMIM5	1.2E-05		1.585	Up	*WNT3*	2.5E-04	5.3E-03		Down
	SNAI1	5.8E-05		1.608	Up	*ZBED6CL*	3.9E-03		1.279	Up
	SNTB1	8.6E-05	5.4E-03		Down	*ZNF773*	4.4E-09		1.611	Up
	SNX18	1.2E-05	2.8E-03	-1.660	Down	*ZNF813*	7.4E-05	4.0E-03		Up
	SPAG4	2.1E-16	6.1E-04	1.738	Up	*ZYG11A*	1.65E-08		2.002	Up
	SRPX	2.31E-04		-1.345	Down					
	SSFA2	1.3E-04		1.510	Up					
	STEAP3	4.6E-05	5.2E-03		Down					
	STX1A	7.1E-07	1.1E-03	1.291	Up					
	SYDE1	3.5E-12	6.6E-04	1.634	Up					
	SYN3	8.3E-06	3.2E-03		Up					
	TBC1D28	8.9E-18	5.6E-04		Up					
	TECR	5.2E-05		1.617	Up					
	TENM4	3.5E-09	8.9E-03		Up					
	TET3	1.8E-07	4.5E-03	1.324	Up					
	TFCP2L1	1.3E-04	6.5E-03	-1.506	Down					
	TGFB1	1.9E-06		1.836	Up					
	TGFBR2	3.8E-05	7.2E-03		Down					
	TIMP3	2.8E-06	1.6E-03		Up					
	TMEM132C	1.3E-05	9.6E-03		Down					
	TMEM173	4.3E-04	6.5E-03		Down					
	TMEM179B	2.2E-04	8.7E-03		Up					
	TNFAIP8L1	4.3E-06	1.2E-03		Up					
	TNFAIP8L2	3.2E-05	7.1E-03		Down					

Table 2.4 Continued …

	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect
IUGR	*AC144568.2*	1.1E-05		1.441	Up	*FAM83F*	2.0E-03	4.3E-03		Up
	AL645730.2	4.7E-03	2.5E-03		Up	*FANCL*	4.7E-05	2.2E-04		Down
	ANP32A	4.4E-04	1.8E-03		Down	*FAT2*	1.2E-04	2.4E-03		Up
	AP000974.1	1.6E-03	4.5E-03		Up	*FBXL5*	6.0E-05	3.7E-05		Down
	ARHGAP29	9.81E-03		-1.949	Down	*FGF13*	3.6E-09	4.4E-06	-1.694	Down
	ARL15	1.19E-03		-1.537	Down	*FSTL3*	5.0E-13	4.8E-06		Up
	BMP1	4.0E-04	4.2E-04		Up	*FUT3*	3.4E-07	2.1E-05		Up
	C1QTNF6	8.3E-05		1.535	Up	*GABRP*	1.8E-04	2.9E-04	1.313	Up
	C5orf27	1.5E-07	8.9E-04		Up	*GLRX*	1.5E-04	3.2E-04		Up
	CALHM2	1.1E-03	3.7E-03		Up	*GPAM*	2.8E-04	2.4E-04		Down
	CALM2	3.59E-03		-1.833	Down	*GPR34*	1.1E-09	2.9E-06	-2.019	Down
	CCDC25	7.29E-03		-1.308	Down	*GSR*	4.4E-04	3.0E-04		Down
	CDV3	5.36E-03		-1.699	Down	*HAPLN3*	1.2E-04	3.7E-04		Up
	CEACAM19	3.4E-06		1.578	Up	HMGN2	1.1E-04		-1.830	Down
	CEACAM7	2.3E-22	6.2E-05		Up	*HPGDS*	8.2E-06	8.7E-03		Down
	CERCAM	2.9E-03		1.576	Up	*HTRA1*	7.5E-07		1.540	Up
	CFL2	1.0E-04		-1.614	Down	IDH1	1.2E-04		-1.611	Down
	CHD3	8.8E-03		1.424	Up	*ILDR2*	6.5E-04	2.9E-04		Up
	CILP	6.8E-04	4.9E-04		Up	*INHA*	3.7E-13	2.2E-07		Up
	CLSTN3	7.0E-04	1.8E-03		Up	*ITGA5*	5.1E-06	6.3E-05		Up
	CLTB	1.4E-06	2.4E-05	1.972	Up	*KAZN*	2.9E-04	3.4E-04		Up
	CSNK1E	1.9E-04	3.2E-03		Up	*KIAA1328*	3.9E-04	2.8E-03		Up
	CST6	3.8E-17		1.816	Up	*KIF17*	2.5E-04		1.351	Up
	CTD-2583A14.10	8.7E-05	3.7E-05	1.605	Up	LANCL1	5.8E-03		-1.858	Down
	CYorf17	3.6E-03	1.4E-05		Down	LIX1L	1.9E-03		-1.708	Down
	DACH1	3.2E-04	4.3E-03		Down	*LPCAT1*	6.8E-03		1.222	Up
	DGKZ	2.8E-04		1.898	Up	LPHN2	3.0E-03		-1.621	Down
	DSN1	9.8E-04	4.6E-04		Down	MAF	1.6E-03		-1.496	Down
	DVL1	4.4E-04		1.913	Up	MAP7D2	7.5E-04		-1.290	Down
	EIF3E	1.2E-04		-2.050	Down	MED21	6.4E-04		-1.512	Down
	EXOC6	2.4E-03		-2.176	Down	*MEOX2*	1.8E-03	1.5E-04		Down
	EXPH5	4.1E-03	2.9E-03		Up	METTL7A	4.0E-04		-1.500	Down
	F13A1	8.6E-04		-1.773	Down	*MOK*	3.8E-09	9.2E-05	1.731	Up
	FABP5	8.1E-03	2.1E-03		Down	*MPRIP*	4.9E-03		1.705	Up
	FAM101B	6.7E-05	8.7E-05	-1.408	Down	*MS4A4E*	1.4E-05	8.2E-05		Up
	FAM134B	1.6E-03		-1.525	Down	*MUC1*	1.8E-11		1.664	Up
	FAM26D	1.3E-05	2.2E-04		Down	*MYH10*	1.1E-04	3.4E-05		Down

	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect
IUGR	*NEURL1*	6.7E-07	8.2E-05	1.642	Up	SLC26A2	0.000430		-1.593	Down
	NFATC2	3.8E-03		-1.677	Down	SLC35D1	0.000202		-1.730	Down
	NOS2	1.1E-05	1.2E-06		Up	*SLC7A8*	7.8E-04	1.9E-04		Up
	NPAS2	3.2E-04	2.8E-05		Up	SLC9A9	0.001185		-1.520	Down
	NPL	1.5E-03		-1.407	Down	*SLCO2A1*	8.2E-06	2.4E-04		Up
	NTF4	2.5E-06	1.0E-04		Up	*SMIM5*	1.1E-06	2.6E-05	1.477	Up
	ODF3B	4.3E-07		1.439	Up	*SMIM6*	4.1E-05		1.414	Up
	PBX1	1.6E-03	2.3E-04		Down	*SNX18*	4.08E-05	4.4E-05	-1.644	Down
	PDE3B	1.5E-03		-1.484	Down	SNX2	0.000041		-1.644	Down
	PIGF	4.9E-03		-1.652	Down	*SPATA13*	4.3E-03	1.5E-04		Up
	PITPNM2	5.0E-04	3.9E-05		Up	*SRSF5*	4.7E-03		1.476	Up
	POLA1	3.1E-04	9.7E-05		Down	*STAT5A*	1.2E-04	4.4E-05		Up
	PPME1	4.1E-05	3.9E-06		Up	STXBP6	0.005398		-1.613	Down
	PRKG1	1.8E-03		-1.544	Down	SYCP2	0.001336		-1.595	Down
	PROSC	1.3E-03		-1.445	Down	*TCHH*	1.0E-04	1.6E-05		Up
	PTDSS1	7.5E-04	5.8E-05		Down	*TFAP2E*	4.88E-04	2.5E-04		Down
	PTGFRN	8.0E-05	2.1E-04		Up	*TGFB1*	3.2E-09	8.5E-05	2.004	Up
	PTPRF	3.6E-04	1.8E-04		Up	*TGM2*	1.1E-03	1.6E-04		Up
	PWWP2B	1.1E-03		1.449	Up	TLR1	0.000303		-1.353	Down
	QSOX1	5.0E-13	8.0E-11	1.636	Up	TLR7	0.007157		-1.280	Down
	RANBP17	1.7E-04		-1.449	Down	*TM4SF18*	5.40E-03	1.6E-04		Down
	RBBP7	6.0E-03		-1.263	Down	*TMC6*	4.2E-05	1.1E-04		Up
	RBMS3	7.7E-03		-1.546	Down	*TMEM132A*	4.2E-04	1.5E-04	1.315	Up
	RBPJ	9.0E-03		-1.604	Down	*TMEM184A*	5.3E-04		1.409	Up
	RHOD	1.7E-04		1.394	Up	*TOX*	2.77E-03	9.4E-05		Down
	RHOF	1.2E-04		1.474	Up	TRMT5	0.003128		-1.238	Down
	RP11-307N16.6	1.3E-04	4.7E-05		Up	UQCRB	0.003594		-1.689	Down
	RP11-544M22.13	5.5E-03		1.512	Up	*VANGL2*	2.05E-03	9.2E-05		Down
	SATB1	4.7E-03		-1.559	Down	*VARS2*	8.2E-06	3.8E-06		Up
	SCN7A	1.8E-06		-1.702	Down	*VN1R2*	6.7E-05	9.0E-05		Up
	SEMA6D	3.9E-03		-1.491	Down	*VTCN1*	1.7E-05	5.3E-08		Up
	SFT2D2	1.3E-03	2.3E-04		Up	*VWCE*	4.0E-08	1.9E-04		Up
	SH2D5	5.6E-05	1.1E-04	1.375	Up	*ZBED6CL*	2.8E-04		1.513	Up
	SH3BGRL	8.7E-06		-1.980	Down	*ZFYVE27*	1.7E-06	7.8E-06		Up
	SH3BGRL2	9.1E-03		-1.645	Down					
	SLC1A6	1.0E-14	1.1E-08		Up					
	SLC22A4	1.3E-03	8.9E-05		Up					

Table 2.4 Continued …

	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect
PE+IUGR	*ABCD1*	2.0E-04	7.4E-03	1.394	Up	*ARRB2*	6.3E-04		-1.666	Down
	ABHD3	3.9E-07	2.3E-04		Down	*ARSJ*	3.4E-06		-1.629	Down
	AC037459.4	1.8E-05	3.1E-03	1.339	Up	*ASPHD2*	7.4E-09	2.8E-04		Up
	AC117395.1	1.7E-03	6.3E-03		Up	*ATP8B4*	8.1E-08	2.0E-03	-1.760	Down
	ACER2	4.2E-05	7.7E-03		Up	*B3GALNT1*	2.2E-03		-1.478	Down
	ACER3	1.5E-04	7.3E-03		Up	*B3GNT2*	6.1E-05	6.2E-03	-2.090	Down
	ACKR2	4.0E-08	4.7E-05	1.910	Up	*B4GALNT2*	8.7E-14	4.7E-03	1.282	Up
	ACTA2	1.6E-05	1.2E-03		Down	*BCAR3*	3.9E-05	6.2E-03		Up
	ADAM10	2.0E-06	1.8E-04		Up	*BCAS4*	1.4E-03		-1.812	Down
	ADAM12	6.4E-12	1.0E-06	1.411	Up	*BCL2L11*	3.7E-08	4.6E-05	2.013	Up
	ADAM2	3.0E-11	3.1E-04		Up	*BCL6*	1.2E-10	5.5E-05	2.051	Up
	ADAMTS1	2.2E-05		-1.537	Down	*BHLHE41*	1.5E-09	6.9E-05	-1.325	Down
	ADAMTS5	7.9E-04		-1.323	Down	*BICC1*	1.3E-05		-1.668	Down
	ADH5	3.5E-05	2.3E-03		Down	*BIN1*	4.3E-05	8.8E-03	-1.906	Down
	AFAP1	2.6E-05	4.8E-03		Up	*BLOC1S4*	4.0E-05	7.6E-03		Up
	AGMAT	2.2E-06	1.6E-03		Up	*BLVRA*	2.2E-05	2.8E-03		Down
	AIF1	1.5E-04		-1.580	Down	*BMP4*	8.1E-08	1.6E-03	-2.170	Down
	ALDH1A1	2.5E-08		-1.571	Down	*BMX*	1.1E-05	1.5E-03		Down
	ALPK3	1.2E-06	4.5E-03		Up	*BNC2*	8.0E-04		-1.472	Down
	AMIGO2	3.6E-04		-1.527	Down	*BTNL9*	9.0E-15	9.3E-04	2.525	Up
	ANKRD24	2.0E-07	2.6E-03	1.662	Up	*BZW2*	1.5E-05	3.1E-03	1.750	Up
	ANKRD9	2.3E-06	4.6E-04		Up	*C10orf128*	1.9E-05		-1.664	Down
	ANPEP	9.9E-09	7.1E-04	-1.394	Down	*C10orf90*	1.2E-09	3.9E-05	1.416	Up
	ANXA4	4.9E-06	4.3E-03		Up	*C12orf75*	1.6E-12	3.3E-05	2.101	Up
	AP000349.1	1.5E-04	3.4E-03		Up	*C14orf37*	2.3E-08	2.3E-03		Up
	AP3B2	3.8E-06	2.7E-04		Up	*C18orf54*	1.0E-04	3.1E-03		Up
	APLN	2.0E-07	1.2E-03		Down	*C1QA*	2.1E-04		-1.506	Down
	APOL4	3.8E-04	3.7E-03		Up	*C1QTNF6*	1.1E-12	1.7E-09		Up
	AQP1	7.7E-09	3.0E-03		Up	*C2*	2.1E-09	6.8E-05	-1.890	Down
	ARHGAP26	1.8E-05	7.2E-04		Up	*C3AR1*	3.1E-07	5.2E-04	-1.752	Down
	ARHGAP9	2.2E-05	9.9E-03	-2.173	Down	*C8orf58*	2.1E-05	6.5E-03		Up
	ARHGEF16	1.1E-03	9.7E-03		Up	*CA8*	3.5E-05	9.6E-03		Down
	ARHGEF4	4.0E-13	1.0E-03	1.684	Up	*CADM3*	1.0E-13	7.7E-03	-1.561	Down
	ARMS2	1.8E-29	1.8E-06	1.611	Up	*CALHM3*	4.4E-04		1.369	Up
	ARNT2	4.9E-13		1.488	Up	*CALM1*	7.1E-08	5.7E-04		Up

	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect
PE+IUGR	*CARD16*	1.5E-03		-1.608	Down	*CP*	2.7E-07		1.272	Up
	CARKD	3.1E-03		1.627	Up	*CPQ*	3.3E-05	3.2E-03	-2.020	Down
	CASP1	3.9E-06	1.1E-03	-2.417	Down	*CPXM1*	4.8E-04		-1.595	Down
	CBLB	3.0E-07	3.1E-04		Up	*CRH*	2.8E-29	3.2E-08	1.646	Up
	CCDC113	2.2E-13	1.3E-08	1.878	Up	*CRIPAK*	1.2E-03	9.3E-03		Up
	CCDC147	7.2E-07	2.7E-04		Up	*CRYBB1*	1.5E-04		-1.723	Down
	CCDC149	8.4E-06	2.5E-03	-1.645	Down	*CSF1*	9.8E-05	9.9E-03	-1.739	Down
	CCDC152	6.1E-04		-1.713	Down	*CSF2RB*	4.7E-06	3.6E-03	1.887	Up
	CCDC183	2.7E-16	2.8E-06	1.817	Up	*CSNK2A2*	4.5E-05	6.9E-03	1.876	Up
	CCDC94	8.9E-07	3.1E-04	1.359	Up	*CSRP2*	4.6E-04	4.6E-03		Down
	CCDC96	1.6E-07	1.6E-04		Up	*CST6*	1.1E-21	7.3E-05	2.075	Up
	CCL28	7.9E-06	3.3E-04		Up	*CTB-60B18.6*	1.5E-05		1.338	Up
	CCR7	4.1E-06		1.428	Up	*CTSC*	4.3E-08	1.0E-04	-1.967	Down
	CCSAP	7.6E-08	1.1E-03		Up	*CTTNBP2*	1.1E-04		-1.456	Down
	CD163	2.9E-07	1.8E-03		Down	*CXXC5*	8.1E-03		1.223	Up
	CD200R1	5.1E-05		-1.663	Down	*CYBB*	4.6E-09	3.4E-04	-1.612	Down
	CD28	6.1E-05		-1.615	Down	*CYP11A1*	5.2E-07	8.1E-04	1.660	Up
	CD33	2.9E-04		-1.723	Down	*CYP26A1*	8.4E-11	2.1E-04		Up
	CD4	1.9E-04		-2.137	Down	*CYP8B1*	7.7E-05	6.6E-03	1.513	Up
	CD53	6.4E-06	7.0E-03		Down	*CYTH4*	2.8E-04		-1.768	Down
	CD63	9.0E-04		1.683	Up	*DCN*	4.0E-03		-1.798	Down
	CDA	1.4E-08		1.852	Up	*DDA1*	2.5E-04	8.1E-03		Up
	CDK7	4.2E-04	5.4E-03		Down	*DDR1*	1.9E-06	9.8E-04	1.656	Up
	CFL2	2.4E-04		-1.643	Down	*DGKZ*	2.1E-06	5.3E-04	1.941	Up
	CGB	5.6E-06		1.396	Up	*DIO2*	2.8E-18	1.3E-05	1.824	Up
	CHAD	2.1E-03		1.632	Up	*DLG2*	1.7E-07	6.6E-04		Down
	CHPT1	4.3E-08	1.2E-05		Down	*DNAH11*	5.9E-08	3.1E-03		Up
	CLDN6	1.8E-08	2.4E-03	1.636	Up	*DNM2*	1.8E-07	4.4E-04	2.010	Up
	CLDN9	3.6E-13	3.0E-03	2.018	Up	*DOCK2*	9.7E-05		-1.755	Down
	CLEC2B	2.2E-03	8.1E-03		Down	*DOK3*	2.2E-05		-2.196	Down
	CLIC3	5.2E-03		1.234	Up	*DOPEY2*	1.5E-04	5.4E-03		Up
	CLIP4	1.1E-05	4.7E-04		Up	*DPYSL3*	3.8E-03		-1.609	Down
	CLTB	8.2E-04		1.712	Up	*DSCR4*	4.1E-08	4.0E-03	1.572	Up
	CORO1A	8.5E-08	2.4E-04	-1.899	Down	*DYRK4*	3.5E-03		-1.594	Down
	CORO2A	4.8E-10	3.8E-04	1.593	Up	*EAF1*	5.1E-03		1.405	Up

	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect
PE+IUGR	*EBI3*	4.4E-13	1.9E-06	1.795	Up	*FAP*	1.8E-03		-1.488	Down
	ECI2	1.1E-03	8.2E-03		Down	*FBXO22*	8.4E-06	7.1E-03	1.641	Up
	EDEM2	1.3E-05	3.3E-03	2.147	Up	*FCER1G*	2.5E-08	3.7E-04	-2.170	Down
	EDIL3	4.6E-07	3.7E-03	-1.491	Down	*FGD6*	5.7E-03		1.741	Up
	EFHD1	2.9E-08	4.9E-04	1.679	Up	*FGF10*	5.3E-10		-1.653	Down
	EFNB1	1.0E-03		1.558	Up	*FGF13*	1.0E-03		-1.783	Down
	EFS	6.8E-03		1.351	Up	*FGL2*	2.6E-09	1.1E-04	-1.355	Down
	EGFL6	3.3E-03		-2.062	Down	*FILIP1L*	1.9E-03		-1.405	Down
	EIF4H	3.2E-03		1.612	Up	*FKBP2*	3.3E-05	2.8E-03	1.341	Up
	ELL	9.9E-03		1.291	Up	*FLNB*	2.6E-07	6.9E-03	1.437	Up
	EML6	2.1E-03		-1.323	Down	*FLT1*	1.0E-30	1.7E-09	3.010	Up
	ENG	1.3E-20	2.8E-10	2.314	Up	*FMN1*	2.3E-06	4.6E-04	1.625	Up
	ENTPD1	3.9E-04		1.466	Up	*FMNL1*	2.8E-04		1.199	Up
	EPHA1	9.8E-05		1.567	Up	*FOLR2*	1.3E-06	7.1E-03	-2.237	Down
	EPS8L1	1.0E-06	6.7E-04		Up	*FOXJ3*	7.6E-05		1.865	Up
	ERGIC1	2.7E-06	1.5E-03	1.729	Up	*FOXRED2*	2.7E-03		-1.508	Down
	ERO1L	9.4E-07	9.5E-03	1.512	Up	*FPR1*	2.7E-04		-2.141	Down
	ERRFI1	2.3E-07	7.3E-03		Up	*FSTL3*	8.0E-28	1.6E-04	1.922	Up
	EZR	1.5E-04		1.833	Up	*FZD5*	1.4E-04	3.0E-03		Down
	F13A1	7.0E-11	6.9E-05	-2.083	Down	*GABRP*	6.2E-04		1.313	Up
	F3	7.9E-05		-1.461	Down	*GALE*	3.2E-04		1.286	Up
	FAM101B	1.4E-10	2.1E-05	-2.303	Down	*GAS1*	6.0E-07		-1.831	Down
	FAM105A	1.7E-05	7.8E-03		Down	*GATA3*	6.6E-05	7.8E-03	1.451	Up
	FAM120A	2.3E-03		1.561	Up	*GBA*	1.9E-09	4.8E-03	1.878	Up
	FAM120AOS	1.5E-06	4.2E-04	1.589	Up	*GFOD2*	5.1E-06	1.4E-03	1.574	Up
	FAM124B	4.7E-04	9.7E-03		Up	*GJA1*	3.7E-04		-1.972	Down
	FAM129B	5.1E-03		1.303	Up	*GLB1*	1.2E-03		-1.509	Down
	FAM156A	5.0E-07	1.3E-04		Up	*GLG1*	6.6E-03		1.398	Up
	FAM156B	6.0E-07	1.0E-04		Up	*GLIS3*	2.3E-08	3.5E-03	1.706	Up
	FAM168B	1.5E-03		1.607	Up	*GLRX*	2.3E-06	8.0E-04	2.106	Up
	FAM198B	1.8E-08	8.8E-04		Down	*GNA15*	3.2E-07	6.8E-04	-2.132	Down
	FAM20A	2.2E-06		-1.803	Down	*GNG2*	9.1E-06		-1.638	Down
	FAM26D	5.5E-09	2.3E-03		Down	*GPIHBP1*	1.4E-10		1.628	Up
	FAM46A	9.1E-05	4.8E-03		Up	*GPT2*	4.7E-09	2.8E-04		Up
	FAM63B	3.3E-04	7.2E-03		Up	*GRHL1*	1.2E-04		1.703	Up

	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect
PE+IUGR	*GRIN2C*	4.3E-05		-1.490	Down	*INSIG1*	1.0E-08	7.0E-05		Up
	GSE1	3.8E-06	7.2E-04	1.767	Up	*INSL4*	1.1E-04		1.297	Up
	GSR	1.1E-03		-1.364	Down	*IQSEC1*	5.9E-04	7.4E-03		Up
	H1F0	6.5E-03		1.833	Up	*ITGA5*	1.6E-07	1.8E-04	1.792	Up
	HAVCR2	4.1E-04		-1.437	Down	*ITGAM*	2.7E-07	3.6E-04	-1.828	Down
	HBEGF	9.0E-05		-1.507	Down	*KCNF1*	5.7E-26	2.3E-07		Up
	HCK	4.6E-05		-1.984	Down	*KCNJ5*	1.2E-05		-1.923	Down
	HCST	1.2E-04		-1.939	Down	*KCNQ1*	6.1E-05	5.1E-03	-1.678	Down
	HEXB	5.4E-12	8.0E-05	2.518	Up	*KIAA0226L*	1.1E-07	5.2E-04		Down
	HHEX	9.4E-05	3.7E-03		Down	*KIF17*	1.7E-07	5.0E-03	1.816	Up
	HK2	6.4E-12	5.6E-03		Up	*KIF2A*	1.1E-07	1.1E-03		Up
	HMGB3	1.1E-04		1.476	Up	*KL*	9.0E-03		-1.439	Down
	HMHA1	1.6E-12	6.7E-05		Up	*KRT15*	1.2E-04	4.4E-03		Up
	HNMT	2.0E-04		-1.790	Down	*KRT19*	2.4E-08	2.3E-03	1.355	Up
	HPGDS	2.3E-08	1.0E-03		Down	*LAD1*	5.3E-03		1.149	Up
	HSD17B2	3.4E-03		-1.526	Down	*LAIR1*	2.3E-06	5.7E-03	-1.692	Down
	HSD3B1	5.5E-03		1.309	Up	*LAMA2*	3.3E-04		-1.872	Down
	HSPA12B	6.3E-04	9.3E-03		Up	*LAMC1*	9.1E-05		-1.774	Down
	HTRA1	8.8E-16	1.2E-04	2.231	Up	*LARGE*	5.4E-05	1.8E-03		Down
	HTRA4	1.2E-33	1.2E-11	2.536	Up	*LARP4B*	6.7E-04	5.2E-03		Up
	ICK	1.2E-04	4.2E-03		Up	*LCP2*	1.5E-05	1.2E-03	-1.978	Down
	IDH1	4.0E-08	2.8E-05	-1.749	Down	*LEP*	4.2E-25	2.8E-06	2.180	Up
	IGDCC4	1.3E-05	5.5E-04		Up	*LFNG*	4.6E-05	1.3E-03		Up
	IGSF3	1.1E-06	9.8E-04	1.735	Up	*LHX6*	3.1E-03	9.3E-03		Up
	IHH	1.6E-04	4.7E-03		Up	*LIMCH1*	1.5E-13	1.3E-06	1.589	Up
	IL12RB2	2.6E-08	2.4E-03	-1.658	Down	*LIMD1*	2.3E-08	1.4E-04		Up
	IL1RAP	3.3E-06	3.8E-03	1.534	Up	*LIMK1*	1.1E-03		1.858	Up
	IL20RA	4.6E-06	4.1E-03		Up	*LPAR5*	1.9E-06	2.0E-03	-1.858	Down
	IL2RB	1.3E-06	1.1E-03		Up	*LRIG3*	1.1E-04	7.1E-03		Down
	ILDR2	2.0E-05	3.1E-03	1.724	Up	*LRP3*	1.8E-03		-1.805	Down
	ILVBL	1.4E-10	8.8E-07	1.540	Up	*LRRC1*	2.1E-07	3.1E-03		Up
	IMPA2	4.0E-03		1.576	Up	*LRRC4*	1.9E-09	2.1E-04	-1.725	Down
	INHA	5.5E-26	7.2E-08	2.270	Up	*LTF*	1.4E-13	7.7E-03		Up
	INHBA	2.3E-21	7.0E-06	1.885	Up	*LY6D*	2.4E-15		1.553	Up
	INPP5B	5.1E-03		1.578	Up	*LYN*	1.6E-06	9.2E-04		Up

Table 2.4 Continued …

	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect
PE+IUGR	*LYVE1*	4.2E-08	5.4E-04	-1.887	Down	*NDRG1*	5.2E-11	9.2E-05	1.468	Up
	MAF	3.1E-06	1.8E-03	-1.962	Down	*NEK11*	4.1E-13	2.1E-04	1.689	Up
	MAFF	1.6E-07	5.0E-05		Up	*NEURL1*	2.8E-09	3.1E-04	1.834	Up
	MAFK	1.5E-06	1.0E-04		Up	*NFASC*	3.1E-15	2.7E-03	-1.372	Down
	MAN1A1	6.9E-03		-1.445	Down	*NFATC2*	9.6E-04		-1.606	Down
	MAN1C1	1.7E-08	4.0E-05	1.401	Up	*NLRP2*	5.2E-03		2.125	Up
	MAP3K3	6.6E-03		-1.378	Down	*NMUR1*	2.2E-05	1.5E-03		Up
	MAP4	9.4E-03		-1.295	Down	*NOG*	3.7E-10	3.1E-04		Up
	MAPK13	2.7E-03		1.615	Up	*NOS2*	2.8E-05	4.2E-03		Up
	MAST4	2.3E-05	5.2E-03	1.883	Up	*NPL*	3.3E-05		-1.576	Down
	MAT2A	4.7E-03		-1.779	Down	*NR2F1*	3.0E-06	6.9E-03		Down
	MATN2	9.3E-03		-1.803	Down	*NRIP1*	2.2E-06	3.3E-03		Up
	MBD2	3.7E-04		1.720	Up	*NRP2*	5.8E-05	7.3E-03		Down
	MCFD2	2.6E-04	9.9E-03		Up	*NTF4*	1.5E-04		1.714	Up
	MCM6	6.7E-05	1.9E-03		Down	*NTRK2*	3.6E-26	2.7E-08	2.082	Up
	MED12L	3.2E-07	3.7E-05		Up	*NUDT16*	5.7E-03		-1.634	Down
	MED13L	1.1E-03		2.079	Up	*OAT*	3.5E-04	6.5E-03		Down
	MEGF10	1.7E-07	3.3E-04		Down	*OCRL*	2.3E-05	3.9E-03	1.811	Up
	METTL7B	2.7E-04		-1.668	Down	*ODF2L*	3.4E-05	9.8E-04		Up
	MFAP5	4.9E-10	1.3E-05	2.221	Up	*OLAH*	2.4E-05	1.3E-03		Up
	MICAL3	4.5E-06	4.4E-03	1.487	Up	*OLFML2B*	3.2E-04	6.6E-03		Down
	MKNK1	1.8E-03		-1.608	Down	*OLFML3*	8.4E-12		-2.009	Down
	MMP11	8.2E-05	2.0E-03		Up	*OR2T33*	4.9E-04	7.0E-03		Up
	MORN3	6.1E-05		1.356	Up	*OR2T8*	2.7E-05	7.0E-03		Up
	MPZL1	1.5E-04	4.1E-03		Down	*OSR1*	4.2E-08	3.2E-03	-1.625	Down
	MRC1	1.0E-07	9.2E-04	-1.640	Down	*PADI1*	1.3E-13	1.3E-05		Up
	MRC1L1	2.4E-07	9.8E-04	-1.571	Down	*PAFAH2*	3.4E-05	3.1E-04		Up
	MS4A6A	1.1E-05	3.1E-03	-1.756	Down	*PAK3*	1.4E-07	1.0E-04		Up
	MTMR4	1.1E-09	3.4E-06	1.472	Up	*PAM*	1.1E-10	1.3E-06	1.589	Up
	MYO15A	5.8E-07	2.6E-04		Up	*PAPPA2*	4.6E-14	8.7E-05	1.825	Up
	MYO7B	5.6E-21	2.7E-07	2.319	Up	*PCDH17*	6.9E-04	1.8E-03		Down
	N4BP3	1.6E-05	7.1E-03		Up	*PDGFC*	3.3E-03		-1.707	Down
	NCCRP1	4.3E-11	7.7E-05	1.883	Up	*PDPN*	3.2E-05		-1.928	Down
	NCKAP1L	7.8E-07	2.0E-03	-1.710	Down	*PDZD7*	4.2E-16	2.1E-06	1.884	Up
	NCSTN	9.9E-03		1.837	Up	*PDZRN3*	6.6E-05		-1.740	Down

	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect
PE+IUGR	*PGD*	1.0E-06	2.0E-04		Down	*PVRL4*	1.7E-12	5.9E-05	1.820	Up
	PHKA2	1.1E-03		1.508	Up	*PYGL*	2.7E-04	3.0E-03		Down
	PHYHIP	1.1E-15	3.6E-06		Up	*QPCT*	4.7E-17	2.1E-05	2.251	Up
	PIK3AP1	5.2E-12	3.7E-07	2.141	Up	*QSOX1*	7.5E-05		1.519	Up
	PIK3CB	8.2E-08	1.4E-04	2.289	Up	*RAB25*	1.0E-03		1.539	Up
	PIK3CD	3.4E-05	4.7E-03	-1.896	Down	*RAB3IL1*	2.8E-10	3.7E-05	-2.637	Down
	PJA1	1.1E-04	4.2E-03		Up	*RAB7L1*	2.0E-04	3.7E-03		Down
	PLA2G15	3.5E-05	1.5E-03		Down	*RAET1L*	1.3E-04	9.7E-03		Up
	PLA2G16	5.2E-13	1.0E-06	1.602	Up	*RALYL*	1.5E-09		-1.544	Down
	PLCB2	2.4E-05		-1.773	Down	*RASEF*	4.8E-07	2.5E-04	1.639	Up
	PLCG2	6.2E-04		-1.609	Down	*RASSF7*	2.3E-07	4.2E-04	1.484	Up
	PLD4	3.9E-06		-1.199	Down	*RASSF8*	2.2E-05	7.4E-03	2.130	Up
	PLEC	3.1E-07	1.2E-04		Up	*RBP1*	1.4E-04		-1.523	Down
	PLEK	1.3E-05		-1.838	Down	*RCAN1*	1.0E-04	6.7E-04		Down
	PLEK2	2.6E-05	2.5E-03		Down	*RCC2*	1.5E-04	6.7E-03		Down
	PLEKHA6	1.5E-09	4.0E-06	1.407	Up	*RDH13*	3.4E-16	5.7E-09	1.725	Up
	PLTP	1.5E-04		-1.514	Down	*RECQL5*	1.9E-04	4.5E-03	1.447	Up
	PMP22	1.6E-05		-1.735	Down	*RFK*	6.7E-06		1.686	Up
	PNCK	5.4E-27	5.7E-09	1.938	Up	*RGAG4*	3.0E-03		-1.489	Down
	POR	5.1E-04	9.7E-03		Up	*RGL1*	9.7E-04		-1.544	Down
	PPIL2	4.6E-04		1.432	Up	*RHBDD2*	3.1E-06	3.3E-04		Up
	PPP1R1C	1.0E-14	1.9E-06		Up	*RNF130*	7.3E-03		-1.350	Down
	PRKCH	2.5E-03		1.638	Up	*RNF223*	2.4E-15	3.1E-06		Up
	PRKG1	7.2E-03		-1.623	Down	*ROR1*	3.6E-05	6.1E-03		Down
	PROCR	4.8E-16	6.5E-07	2.427	Up	*RP11-211G3.3*	2.7E-06	3.6E-03		Up
	PRPS2	1.0E-03		-1.701	Down	*RP11-986E7.7*	7.0E-15	7.6E-08	1.547	Up
	PRRG1	2.3E-07	6.6E-04	1.832	Up	*RREB1*	2.7E-04		1.701	Up
	PRRX1	2.2E-11		-1.770	Down	*RTN4RL1*	1.0E-08	4.0E-03		Down
	PRSS54	4.6E-11	3.5E-05	1.705	Up	*RTN4RL2*	2.6E-05	2.4E-03		Up
	PSG11	5.4E-04		1.523	Up	*RYBP*	5.9E-07	6.1E-05		Up
	PTDSS1	7.1E-04	6.9E-03		Down	*RYR1*	1.6E-06		-1.703	Down
	PTGS1	2.2E-05	6.5E-03		Down	*SAMSN1*	7.2E-05		-1.520	Down
	PTPN9	3.5E-03		-1.353	Down	*SASH1*	3.9E-11	1.4E-04	1.861	Up
	PTPRF	3.3E-04		1.330	Up	*SASH3*	5.3E-05		-2.008	Down
	PTPRO	1.5E-06	1.3E-03		Down	*SCARB1*	4.9E-08	8.2E-04	1.904	Up

	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect
PE+IUGR	*SCN9A*	2.8E-08		-1.745	Down	*SLC7A1*	1.9E-07	2.7E-04		Up
	SCUBE2	1.1E-11	9.7E-03	-1.762	Down	*SLC7A7*	7.1E-05		-1.725	Down
	SDC3	2.4E-10	1.3E-06	1.677	Up	*SLC9A9*	7.9E-11	8.5E-06	-2.045	Down
	SEL1L3	1.8E-04	8.9E-03		Down	*SLCO2A1*	7.0E-12	7.7E-05	1.916	Up
	SELE	1.7E-07	4.4E-04		Down	*SLIT2*	4.3E-05		-1.896	Down
	SELPLG	5.2E-03		-1.619	Down	*SMARCA1*	1.9E-07	2.3E-04	1.403	Up
	SEPP1	8.5E-04		-1.953	Down	*SMIM14*	2.2E-04	7.6E-03		Up
	SERINC5	3.0E-04	3.2E-03		Up	*SMIM5*	2.6E-08	7.4E-05	2.058	Up
	SERPINA3	1.8E-19	1.5E-10		Up	*SNX10*	1.1E-04		1.390	Up
	SERPINF1	3.9E-06	2.5E-03		Down	*SNX11*	1.0E-03	6.4E-03		Down
	SFT2D2	5.2E-04	4.4E-03		Up	*SNX18*	3.9E-07	1.5E-04	-2.139	Down
	SFXN3	4.5E-15	1.3E-09	1.567	Up	*SNX20*	1.0E-03		-1.573	Down
	SGPP1	2.4E-03		1.371	Up	*SNX9*	9.1E-06	5.0E-03	1.519	Up
	SH2D5	4.2E-07	6.1E-05	1.379	Up	*SORBS2*	2.7E-03		-1.724	Down
	SH3BP2	5.3E-06	2.4E-04		Up	*SOWAHC*	2.2E-05	3.8E-03		Up
	SH3BP5	2.7E-13	3.8E-05	1.776	Up	*SPAG4*	5.7E-16	1.0E-04	1.689	Up
	SH3D21	5.9E-05	1.5E-03	1.471	Up	*SPI1*	3.5E-05	7.8E-03	-1.968	Down
	SH3PXD2A	1.7E-09	1.2E-04	2.211	Up	*SPTLC3*	2.5E-07	1.7E-03		Up
	SHANK2	4.7E-04		1.582	Up	*SREBF1*	1.7E-09	7.4E-06	1.458	Up
	SIGLEC1	2.6E-04		-1.731	Down	*SRPX*	6.0E-09	7.1E-03	-2.384	Down
	SIGLEC6	1.2E-13	6.8E-05	1.557	Up	*SRSF5*	2.2E-07	8.2E-05		Up
	SIPA1L1	2.0E-06	7.1E-04	1.581	Up	*SSFA2*	4.0E-07	1.1E-03	1.997	Up
	SLA	4.8E-03		-1.447	Down	*ST3GAL1*	1.4E-04	8.5E-03		Up
	SLAMF1	4.8E-26	5.0E-05	-2.479	Down	*ST6GAL1*	3.8E-04		-1.643	Down
	SLC11A2	7.5E-04		1.820	Up	*ST8SIA6*	3.2E-07		1.413	Up
	SLC12A7	5.5E-03		-1.782	Down	*STRA6*	6.7E-04	7.4E-03		Up
	SLC16A10	3.4E-07		-1.675	Down	*STS*	3.9E-05	3.7E-03		Up
	SLC16A3	6.2E-07	3.0E-03	1.357	Up	*SYDE1*	1.5E-13	2.8E-06	1.553	Up
	SLC16A5	8.0E-04		1.400	Up	*SYK*	5.7E-05	4.8E-03	-1.494	Down
	SLC1A6	1.0E-14	2.0E-03		Up	*SYNPO*	3.4E-04	7.4E-03		Up
	SLC2A11	4.6E-05	1.8E-03		Up	*SYT12*	2.4E-03		1.312	Up
	SLC2A4	5.7E-05	5.5E-03		Up	*SYTL5*	9.7E-06		-1.683	Down
	SLC35D1	2.5E-06	8.3E-04		Down	*TADA2B*	6.1E-05	4.3E-03		Up
	SLC6A6	1.5E-08	8.2E-05		Up	*TBC1D22A*	4.9E-05	5.4E-03		Up
	SLC6A8	1.1E-13	1.3E-05	1.532	Up	*TBC1D26*	1.5E-08	5.4E-03		Up

	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect	Gene Symbol	DESeq2*	edgeR*	ALDEx2+	Direction of effect
PE+IUGR	*TBC1D28*	2.5E-21	2.0E-07	1.740	Up	*UBE2Q2*	7.3E-08	4.6E-05	2.120	Up
	TBXAS1	2.2E-03		-1.508	Down	*UBE2S*	1.1E-03	6.3E-03		Down
	TCF25	5.7E-05	7.0E-03		Up	*UBR1*	1.2E-05	8.7E-04		Up
	TCHH	5.6E-04	9.3E-04		Up	*UNC5C*	5.5E-04		-1.318	Down
	TECR	4.3E-06	8.7E-04	1.529	Up	*UPK2*	2.4E-10		1.266	Up
	TENM4	9.2E-11		1.714	Up	*UQCRB*	6.7E-03		-1.552	Down
	TET3	2.5E-08	4.7E-04	1.698	Up	*UST*	4.2E-05		-1.523	Down
	TFAP2A	1.1E-05	1.0E-03	1.297	Up	*VANGL2*	4.2E-05		-1.533	Down
	TFPI	2.6E-04		1.615	Up	*VAV1*	3.1E-07	1.1E-03	-2.220	Down
	TGFB1	4.1E-07		1.854	Up	*VEGFC*	5.6E-04	7.7E-03		Up
	TGFBR2	8.4E-04		-1.414	Down	*VGLL3*	3.6E-06	4.7E-04		Up
	TGOLN2	1.3E-03		1.597	Up	*VIT*	1.6E-09		1.402	Up
	TIMD4	3.2E-04	7.6E-03		Down	*VNN1*	2.7E-08	4.5E-03	-1.495	Down
	TLE4	3.6E-06	1.4E-03		Down	*VSIG4*	6.1E-10	1.7E-04	-1.875	Down
	TLR7	1.2E-06	4.3E-03		Down	*VTCN1*	9.1E-07		1.381	Up
	TMEM141	7.5E-05	4.2E-03		Up	*VTN*	3.0E-08		-1.799	Down
	TMEM176B	2.7E-05		-1.739	Down	*VWCE*	7.7E-19	1.7E-06	2.277	Up
	TMEM179B	2.0E-03		1.904	Up	*WAS*	3.2E-04		-1.660	Down
	TMEM184A	3.8E-07	3.8E-05		Up	*WDR45*	1.5E-05	1.1E-03		Up
	TMEM212	3.4E-06	5.5E-05		Up	*WDR90*	4.5E-05	1.3E-03		Up
	TMEM45A	1.0E-07	9.5E-03		Up	*WLS*	1.9E-09	1.4E-05	-1.410	Down
	TMEM98	5.5E-03		-1.627	Down	*WNT2*	1.6E-06		-1.337	Down
	TMSB4X	5.4E-04		-1.927	Down	*YPEL4*	2.8E-08		2.100	Up
	TNFAIP8	2.5E-04	8.4E-03		Up	*ZCCHC2*	1.7E-04		1.552	Up
	TNFAIP8L1	2.3E-03		2.090	Up	*ZMYND8*	6.1E-04		1.670	Up
	TNFRSF11A	5.4E-08	4.8E-03		Down	*ZNF354B*	2.2E-04	1.5E-03		Up
	TNKS1BP1	2.2E-05	1.9E-03		Up	*ZNF443*	3.5E-04	7.7E-03		Down
	TNKS2	4.6E-05	9.8E-04		Up	*ZNF614*	1.7E-07	9.5E-06		Up
	TPBG	4.9E-13	1.7E-04	1.888	Up	*ZNF773*	1.0E-13	7.8E-08		Up
	TRPM2	1.8E-09	8.6E-05	-2.062	Down	*ZSWIM4*	7.7E-05		1.779	Up
	TSPAN11	6.1E-05	5.0E-03		Up	*ZYG11A*	6.3E-12	5.0E-05	1.878	Up
	TTBK2	6.3E-04	3.6E-03		Up					
	TTC17	5.6E-05	7.2E-03		Up					
	TYRO3	8.9E-07	1.0E-03	-1.624	Down					
	UBB	9.7E-04		-1.514	Down					

Table 2.5 List of genes common between: PE Only and PE + IUGR, PE Only and IUGR Only and PE + IUGR and IUGR Only.

PE Only and PE + IUGR				IUGR Only and PE + IUGR	PE Only and IUGR Only
ACKR2	*ERO1L*	*NFATC2*	*SSFA2*	*C1QTNF6*	*CST6*
ADAM12	*ERRFI1*	*NPL*	*SYDE1*	*CFL2*	*DGKZ*
ADH5	*EZR*	*NTRK2*	*TBC1D28*	*CLTB*	*DVL1*
ALPK3	*F13A1*	*OCRL*	*TECR*	*CST6*	*F13A1*
ANKRD24	*FAM101B*	*OSR1*	*TENM4*	*DGKZ*	*FAM101B*
ANPEP	*FAM120AOS*	*PAM*	*TET3*	*F13A1*	*FGF13*
ARHGEF4	*FCER1G*	*PAPPA2*	*TGFB1*	*FAM101B*	*FSTL3*
ARMS2	*FGF13*	*PDZD7*	*TMEM179B*	*FAM26D*	*GLRX*
B4GALNT2	*FKBP2*	*PHYHIP*	*TNFAIP8L1*	*FGF13*	*HTRA1*
BCAR3	*FLNB*	*PJA1*	*TPBG*	*FSTL3*	*IDH1*
BCL6	*FLT1*	*PLA2G16*	*TRPM2*	*GABRP*	*ILDR2*
BHLHE41	*FOLR2*	*PNCK*	*TYRO3*	*GLRX*	*INHA*
BLVRA	*FPR1*	*PPP1R1C*	*VAV1*	*HPGDS*	*ITGA5*
BMX	*FSTL3*	*PROCR*	*VSIG4*	*HTRA1*	*MAP7D2*
BTNL9	*GALE*	*PRSS54*	*VWCE*	*IDH1*	*MYH10*
C10orf90	*GBA*	*PTPN9*	*WLS*	*ILDR2*	*NEURL1*
C12orf75	*GFOD2*	*PVRL4*	*ZNF773*	*INHA*	*NFATC2*
CALM1	*GLRX*	*QPCT*	*ZYG11A*	*ITGA5*	*NPL*
CARKD	*GPIHBP1*	*RAB3IL1*		*KIF17*	*SLC1A6*
CASP1	*GSE1*	*RASSF7*		*MAF*	*SLC35D1*
CCDC113	*HAVCR2*	*RCAN1*		*NEURL1*	*SLC9A9*
CCDC183	*HEXB*	*RDH13*		*NFATC2*	*SLCO2A1*
CD200R1	*HMHA1*	*RHBDD2*		*NOS2*	*SMIM5*
CD63	*HNMT*	*RP11-211G3.3*		*NPL*	*SNX18*
CLDN6	*HTRA1*	*RP11-986E7.7*		*NTF4*	*TGFB1*
CLDN9	*HTRA4*	*SASH1*		*PRKG1*	*VWCE*
CLIC3	*IDH1*	*SASH3*		*PTDSS1*	*ZBED6CL*
CORO1A	*ILDR2*	*SCARB1*		*PTPRF*	
CORO2A	*ILVBL*	*SCUBE2*		*QSOX1*	
CRH	*INHA*	*SDC3*		*SFT2D2*	
CST6	*INHBA*	*SEL1L3*		*SH2D5*	
CTSC	*INPP5B*	*SERPINA3*		*SLC1A6*	
CYBB	*IQSEC1*	*SFXN3*		*SLC9A9*	
CYP26A1	*ITGA5*	*SH3PXD2A*		*SLCO2A1*	
DGKZ	*KCNF1*	*SIGLEC6*		*SMIM5*	
DIO2	*KRT19*	*SIPA1L1*		*SNX18*	
DLG2	*LEP*	*SLAMF1*		*SRSF5*	
DNAH11	*LIMCH1*	*SLC16A3*		*TCHH*	
DNM2	*LPAR5*	*SLC1A6*		*TGFB1*	
DOK3	*MAST4*	*SLC35D1*		*TMEM184A*	
DSCR4	*MED13L*	*SLC6A6*		*UQCRB*	
EAF1	*MFAP5*	*SLC6A8*		*VTCN1*	
EBI3	*MICAL3*	*SLC9A9*		*VWCE*	
EDIL3	*MYO7B*	*SLCO2A1*			
EFHD1	*NCKAP1L*	*SMIM5*			
EFNB1	*NDRG1*	*SNX18*			
ENG	*NEK11*	*SPAG4*			
ERGIC1	*NEURL1*	*SRPX*			

Table 2.6 Identified candidate gene targets based on inverse correlation analysis including correlation coefficients and gene fold changes.

	MicroRNA	Gene	Spearman's Coefficient	BH Adjusted p-value	Gene Fold Change
PE	miR-193b-5p	*CCR1*	-0.8140	2.8E-09	-1.549
	miR-193b-5p	*FGF13*	-0.8194	1.8E-09	-1.601
	miR-193b-5p	*IL12RB2*	-0.7628	7.7E-03	-1.617
	miR-193b-5p	*NTN4*	-0.7567	1.6E-07	-1.842
	miR-193b-5p	*TYRO3*	-0.8514	8.9E-11	-1.516
	miR-210-5p	*C3AR1*	-0.7680	5.4E-03	-1.564
	miR-210-5p	*TYRO3*	-0.7984	9.4E-09	-1.516
	miR-210-5p	*VAV1*	-0.6808	7.7E-06	-1.634
	miR-210-5p	*WNT3*	-0.7798	3.6E-08	-1.667
IUGR	miR-193b-5p	*FGF13*	-0.7747	2.7E-06	-1.716
PE +IUGR	miR-193b-3p	*APLN*	-0.7151	6.6E-06	-2.825
	miR-193b-5p	*APLN*	-0.7967	6.9E-08	-2.825
	miR-193b-5p	*CSF1*	-0.8160	1.7E-08	-1.572
	miR-193b-5p	*FGF13*	-0.8160	1.3E-09	-1.960
	miR-193b-5p	*IL12RB2*	-0.8126	2.2E-08	-1.909
	miR-193b-5p	*NRP2*	-0.6914	1.9E-05	-1.524
	miR-193b-5p	*PTGS1*	-0.7678	4.3E-07	-1.756
	miR-193b-5p	*TLR7*	-0.7521	1.0E-06	-1.856
	miR-193b-5p	*TYRO3*	-0.8597	3.5E-10	-1.680
	miR-210-5p	*APLN*	-0.7858	1.4E-07	-2.825
	miR-210-5p	*C3AR1*	-0.8015	4.9E-08	-1.932
	miR-210-5p	*CSF1*	-0.8023	4.6E-08	-1.572
	miR-210-5p	*ITGAM*	-0.8054	3.7E-08	-1.816
	miR-210-5p	*SELE*	-0.7377	2.2E-06	-3.193
	miR-210-5p	*TYRO3*	-0.8311	5.0E-09	-1.680
	miR-210-5p	*VAV1*	-0.8059	3.6E-08	-1.912
	miR-365a-3p	*FZD5*	-0.5982	4.8E-04	-1.600
	miR-365b-3p	*FZD5*	-0.6342	1.6E-04	-1.600

Table 2.7 Global microRNA expression studies in human placenta from preeclamptic patients.

MicroRNA	Reference
miR-210	Zhou 2016, Zhang 2015, Xu 2014, Betoni 2013, Ishibashi 2012, Enquobahrie 2011, Mayor-Lynn 2011, Zhu 2009, Pineles 2007
miR-193b-3p	Zhou 2016, Xu 2014, Betoni 2013, Ishibashi 2012, Mayor-Lynn 2011
miR-193b-5p	Ishibashi 2012, Zhou 2016
miR-365a-3p	Zhou 2016
miR-365a-5p	No studies identified
miR-365b-3p	Zhou 2016
miR-520a-3p	Ishibashi 2012, Zhou 2016
miR-181a-2-3p	No studies identified
miR-33b-3p	No studies identified
miR-27a-5p	Zhou 2016, Ishibashi 2012

Zhou, X et al. The aberrantly expressed miR-193b-3p contributes to preeclampsia through regulating transforming growth factor-β signaling. Sci Rep 2016; 6: 1–13.

Zhang, C et al. Placental miR-106a approximately 363 cluster is dysregulated in preeclamptic placenta. Placenta. 2015; 36: 250-252.

Xu, P et al. Variations of microRNAs in human placentas and plasma from preeclamptic pregnancy. Hypertension 2014; 63: 1276–1284.

Betoni, JS et al. MicroRNA analysis in placentas from patients with preeclampsia: comparison of new and published results. Hypertens Pregnancy. 2013; 32, 321-339.

Ishibashi, O et al. Hydroxysteroid (17-beta) dehydrogenase 1 is dysregulated by miR-210 and miR-518c that are aberrantly expressed in preeclamptic placentas: a novel marker for predicting preeclampsia. Hypertension. 2012; 59:265-273.

Enquobahrie, DA et al. Placental microRNA expression in pregnancies complicated by preeclampsia. Am J Obstet Gynecol. 2011; 204:178 e112-121.

Mayor-Lynn, K et al. Expression profile of microRNAs and mRNAs in human placentas from pregnancies complicated by preeclampsia and preterm labor. Reprod Sci 2011; 18: 46-56.

Zhu, X et al. Differential expression profile of microRNAs in human placentas from preeclamptic pregnancies vs normal pregnancies. Am J Obstet Gynecol 2009; 200: 661.e1-e7.

Pineles, BL et al. Distinct subsets of microRNAs are expressed differentially in the human placentas of patients with preeclampsia. Am J Obstet Gynecol 2007; 196: 1–6.

Chapter 3

3 The impact of microRNAs on gene target expression and trophoblast cell functions

3.1 Introduction

MicroRNAs are endogenous, single stranded non-coding RNAs that regulate gene expression post-transcriptionally via sequence complementarity that leads to mRNA degradation or impaired translation.[1,2] MiRNAs are transcribed in the nucleus and are then processed in the cytoplasm into mature miRNAs of 20-22 nucleotides in length.[1,2] In the cytoplasm, miRNAs target messenger RNAs (mRNA) via perfect or imperfect base pair complementarity to the 3′ untranslated region (3′UTR) of the mRNA.[1-3] In mammals, miRNAs are predicted to target approximately 60% of all protein-coding genes.[2,3] Studies have shown that miRNAs participate in the regulation of a wide spectrum of cell processes including proliferation, apoptosis, differentiation and stress response.[2,3] MiRNAs are expressed highly in the placenta, and two placenta-specific clusters have been identified on chromosomes 14 and 19.[4,5] Studies have also identified miRNA expression changes in placentae from pregnancy complications such as preeclampsia (PE) and intrauterine growth restriction (IUGR).[6,7] There is also evidence that placental miRNAs can enter maternal circulation during pregnancy, sparking interest in the utility of miRNAs as potential biomarkers for pregnancy associated diseases.[8]

PE is a maternal hypertensive disorder of pregnancy, affecting 2-8% of pregnancies worldwide.[9,10] The underlying pathophysiology of PE is not fully understood however,

studies have shown that placental maldevelopment in early gestation may contribute to the pathogenesis of PE.[10,11] More specifically, the process of trophoblast cell migration and invasion necessary to remodel maternal spiral arteries to accommodate for increased uteroplacental blood flow is thought to be impaired.[10,11] This can result in decreased uteroplacental blood perfusion and subsequently a hypoxic intrauterine environment.[10,11] A subset of patients with PE also develop IUGR, particularly early in gestation defined as early-onset PE (< 34 weeks). IUGR is defined as poor fetal growth *in utero,* where patients present with fetal weight lower than the 10th percentile estimated for gestational age and gender, and can be associated with abnormal Dopplers in fetal and umbilical vessels.[12] PE and IUGR are pregnancy complications with common histopathological features in the placenta.[13,14] In addition, there is evidence to support that the intrauterine environment in both PE and IUGR is relatively hypoxic.[15] Hypoxia has been linked to the upregulation of miR-210, and validated *in vitro*[16,17] Increased expression levels of miR-210 in placental and plasma samples from patients diagnosed with PE have been consistently reported, and is one of the most commonly reported miRNAs in pregnancy complications.[18-20] Genes such as *EFNA3* and *HOXA9* have been validated as targets of miR-210 in cell culture, and upregulation of miR-210 has been linked to impairment of cell functions such as proliferation and migration of trophoblast cells *in vitro*. [17]

We have previously identified miR-210-3p and -5p to be upregulated in placenta from PE pregnancies irrespective of IUGR occurrence (Chapter 2). We have also identified the upregulation of miR-193b-3p and -5p in placentae from pregnancies complicated with early-onset PE only, IUGR only, and PE + IUGR compared to gestational age matched

controls. In the same study, using RNA sequencing we assessed gene expression in the same placental samples. Integration of the two datasets identified candidate gene targets predominantly for miR-210-5p and miR-193b-5p. For miR-210-5p we identified 7 candidate gene targets in patients with early-onset PE + IUGR (*APLN, C3AR1, CSF1, ITGAM, SELE, TYRO3, VAV1*), and 4 candidate gene targets in patients with early-onset PE only (*C3AR1, TYRO3, VAV1, WNT3*). For miR-193b-5p, we identified 8 candidate gene targets in patients with early-onset PE + IUGR (*APLN, CSF1, FGF13, IL12RB2, NRP2, PTGS1, TLR7, TYRO3*), 5 candidate gene targets in patients with early-onset PE only (*CCR1, FGF13, IL12RB2, NTN4, TYRO3*), and 1 candidate gene target in patients with early-onset IUGR (*FGF13*).

The purpose of this study was to investigate the potential impact of miRNAs 210-5p and 193b-5p on identified gene targets and on trophoblast cell funtion *in vitro*. To fulfill this purpose, the objectives were two-fold (i) examine whether miR-210-5p and miR-193b-5p interact with candidate gene targets using luciferase assays *in vitro*, and (ii) if so, overexpress miR-210-5p and miR-193b-5p in the extravillous trophoblast cell line HTR-8/SVneo and assess the impact on target gene expression, migration, and proliferation of cells.

3.2 Methods

3.2.1 Patient Recruitment

Preeclampsia was defined as hypertension (blood pressure > 140/90 mm Hg) and proteinuria (≥ 300 mg in 24 hours).[9,10] Severe PE is often associated with HELLP

syndrome characterized by the onset of: edema, headache, elevated liver enzymes, and low platelet count. Patients diagnosed with PE and HELLP are indicated in Table 3.1. Intrauterine growth restriction was defined as estimated fetal weight below the 10th percentile for gestational age and gender, associated with abnormal umbilical and uterine artery Dopplers.[12] Patients with PE + IUGR presented with criteria aforementioned for both diseases. Only patients diagnosed prior to 34 weeks (early-onset) were included in this study. Patients with preterm labor and no other pregnancy complications before 34 weeks of gestation were recruited as controls. Women with diabetes, gestational diabetes, pre-existing hypertension, obvious chorioamnionitis (status confirmed at delivery), alcohol/drug use, chromosomal or genetic abnormalities, congenital anomalies, or infection were excluded. All women enrolled in this study gave written informed consent for the collection of samples and information. This research was approved by the office of Human Research Ethics at Western University (REB # 102621).

3.2.2 Placental Tissue Sampling

Samples were collected from two central and two peripheral portions of the placenta within 30 minutes of delivery. Central samples were collected within a 5 cm radius from the umbilical cord insertion site and the peripheral samples were collected 2-3 cm from the edge of the placenta. Full depth 1 cm x 1 cm tissue samples were excised, and the maternal decidua was separated from the chorionic villi using gross dissection. In this study, the maternal and fetal components were separated and only the fetal components (chorionic villi) were used for analysis. The tissue samples were flash frozen in liquid nitrogen and stored at -80°C until further analysis.

3.2.3 Reverse Transcription and Real-Time PCR (Placenta Tissue)

Total RNA was isolated from 80-100 mg of tissue samples from each of the four regions of each placenta using the mirVana RNA isolation kit (Life Technologies, Waltham, MA, USA). Sample quantity and quality was checked using the Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Total RNA isolated from central and peripheral samples of each placenta was pooled in equal quantities for one representative total RNA sample from each patient. Total RNA was reversed transcribed using the High Capacity cDNA Synthesis Kit (Life Technologies™, Waltham, MA, USA). Quantification for mRNAs was completed using the TaqMan® fast advanced PCR master mix (Life Technologies, Waltham, MA, USA) in conjunction with TaqMan gene expression assays. GAPDH was used as an endogenous control. All samples were assayed in triplicate and run on the ViiA7™ real-time machine. The $2^{-\Delta\Delta Ct}$ method was used for fold-change analysis. Mann-Whitney U test was used for statistical analysis. APLN (Hs00175572_m1), C3AR1 (Hs00269693_s1), CCR1 (Hs00928897_s1), CSF1 (Hs00174164_m1), FGF13 (Hs00182807_m1), IL12RB2 (Hs00155486_m1), ITGAM (Hs00167304_m1), NRP2 (Hs00187290_m1), NTN4 (Hs00221915_m1), PTGS1 (Hs00377726_m1), SELE (Hs00950409_g1), TLR7 (Hs01933259_s1), TYRO3 (Hs03986773_m1), VAV1 (Hs01041613_m1), WNT3 (Hs00902257_m1).

3.2.4 Target Prediction

A combination of target prediction tools were used to predict targets, software tools include: TargetScan Human (http://www.targetscan.org/vert_70), Diana micro-T CDS

(http://diana.imis.athena-innovation.gr/DianaTools/), miRwalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2), miRDB (http://mirdb.org), miRanda (http://microrna.sanger.ac.uk/targets), and PicTar (https://pictar.mdc-berlin.de).

3.2.5 Luciferase Assays

HTR-8/SVneo cells were cultured in RPMI-1640 media (Gibco, California, USA) supplemented with 10% fetal bovine serum in a 37°C humidified incubator (5% CO_2). Cells were subcultured at a ratio of 1:3 when cells reached 80% confluency. Plasmids containing the firefly luciferase gene and the 3′UTRs of candidate genes APLN (S813689), CSF1(S807015), FGF13 (S808593), ITGAM(S808425), TYRO3 (S808004), C3AR1 (S803358) were obtained from Active Motif (Carlsbad, CA, USA). DharmaFECT Duo transfection reagent (GE Healthcare, Boston, MA, USA) was used to co-transfect the firefly plasmid (100 ng) with hsa-miR-210-5p mimics, or hsa-miR-193b-5p mimics or NC mimics, 100 nM each. Control 3′UTR reporter vectors were also used, empty 3′UTR (100 ng) (S890005) and 3′UTR of GAPDH (100 ng) (S801378). After 24-hour incubation, luciferase activity was measured using the LightSwitch™ Luciferase assay reagent (Active Motif, Carlsbad, CA, USA).

3.2.6 Immunohistochemistry

Whole sections (0.5 cm x 0.5 cm) were harvested from the placenta, extending from the basal plate decidua into the chorionic villus (maternal and fetal sides). Both central (near umbilical cord insertion site) and peripheral (near outer edge of the tissue) sections were collected. The specimens were immediately fixed in 10% formalin for a minimum of 24

hours. Following fixation and washing, tissues were processed, and paraffin embedded. All tissues were then serially sectioned at a thickness of 5 μm using a microtome and mounted onto slides. Slides were then deparaffinized and rehydrated, and antigen retrieval was performed in citrate buffer (pH 6.0). For APLN and FGF13, the antigen retrieval step was not performed. Slides were then blocked with a blocking agent, Background Sniper (BS966, Biocare Medical, Pacheco, CA, USA). The primary antibody was then applied for: APLN (1:15) (ab181786, Abcam), FGF13 (1:1000) (ab153808, Abcam), CSF1 (1:75) (ab52864, Abcam), and ITGAM (1:75) (ab52478, Abcam). Slides were incubated with the primary antibody overnight. The slides were then rinsed and the secondary antibody, ImmPRESS Anti-Rabbit Peroxidase Polymer Detection Kit (MP-7401, Vector Laboratories, Burlington, ON, Canada), was applied. The slides were rinsed again and labeled with a DAB (3,3′–diaminobenzidine) stain (1718096001, St. Louis, MO, USA). Negative control slides underwent the same procedures, with the exception of the application of the primary antibody. Finally, a CAT Hematoxylin (CATHE, Biocare Medical, Pacheco, CA, USA) counterstain was applied to all slides. Imaging was performed using a 200 x total magnification on a Zeiss AxioImager Z1 Microscope using Zen software and an MRc5 camera (Zeiss Canada Ltd., North York, ON).

3.2.7 Cell Culture and Treatment

HTR-8/SVneo cells were cultured in RPMI-1640 media (Gibco, California, USA) with 10% fetal bovine serum in a 24-well plate, at 37°C in 5% CO_2. Cells were subsequently transfected using DharmaFECT 1 transfection reagent (GE Healthcare, Boston, MA, USA). For miR-210-5p experiments cells were transfected with: 50 nM of miR-210-5p

mimics (MC27291), or 100 nM of miR-210-5p inhibitors (MH27291), or respective negative control (NC) mimics (50 nM, 4464058), NC inhibitors (100 nM, 4464076) (ThermoFisher, Waltham, MA, USA). For miR-193b-5p experiments cells were transfected with: 50 nM of miR-193b-5p mimics (MIM0232), 100 nM of miR-193b-5p inhibitors (INH0232), or respective NC mimics (50 nM, MIM9001), NC inhibitors (100 nM, INH9001) (Active Motif, Carlsbad, CA, USA). After transfection, cells were lysed for gene or protein expression analysis or used to measure cell functions.

3.2.8 Reverse Transcription and Real-time PCR (Cells)

HTR-8/SVneo cells were seeded and transfected as described previously. Total RNA was isolated from HTR-8/SVneo cells using Qiagen's RNeasy Mini kit (74104, Qiagen, Germany). Cells were lysed using lysis buffer provided in the kit and further homogenized by passing lysate through a 20-gauge needle. Total RNA is then used for reverse transcription and real-time PCR as described above in Methods section 3.2.3.

3.2.9 Western Blotting

HTR-8/SVneo cells were seeded and transfected as described previously. Cells were then lysed using RIPA buffer containing protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA). Bradford assay was used to assess protein concentration. Lysates were then resolved on SDS-PAGE and transferred to a PDVF membrane using the Bio-Rad Trans-Blot Turbo transfer system (Bio-Rad, Hercules, CA, USA). Membranes were incubated with primary antibodies against APLN (1:200) (sc-293441, SantaCruz Biotechnology), FGF13 (1:500) (ab153808, Abcam), CSF1 (1:500) (sc-365779,

SantaCruz Biotechnology), ITGAM (1:500) (sc-515923, SantaCruz Biotechnology, ab52478, Abcam) or beta-actin (MS1295P, ThermoFisher) at 4°C overnight. Following this, membranes were washed and incubated with horse radish peroxidase (HRP) conjugated secondary antibody (170-6516, Bio-Rad, Hercules, CA, USA). Resolved protein bands were detected using chemiluminescence, and images were taken using the VersaDoc Imaging System (Bio-Rad, Hercules, CA, USA).

3.2.10 Cell Viability Assay

HTR-8/SVneo cells were seeded and transfected as described previously. Cell proliferation was measured using cell proliferation reagent WST-1 (Sigma-Aldrich, St. Louis, MO, USA) according manufacturer's protocol. After 1 hour incubation with the WST-1 reagent, absorbance was measured at 450 nm using Multiskan Ascent plate reader (ThermoFisher, Waltham, MA, USA). Reference wavelength of 650 nm was used, and culture medium was used as a blank.

3.2.11 Wound Healing (scratch) Assay

An *in vitro* scratch assay was used as described previously. [21] After transfection, HTR-8/SVneo cells were grown to confluence, and scratches were made using a p200 pipette tip. The width of the scratch was monitored by Leica DM IL microscope, images were taken at 0 hours and 24 hours using 40 x total magnification. Width of the scratch was then measured using Image J Software, distance travelled is shown as migration level relative to control samples.

3.2.12 Transwell Migration Assay

Transwell compartments were prepared in a 24-well plate format, with BD Falcon™ 8.0-μm pore Transwell cell culture inserts (353097; BD Biosciences, Franklin lakes, NJ, USA). For the lower compartment 0.8 mL of RPMI-1640 media with 10% FBS was added. For the upper compartment, 1 x 10^5 transfected cells in 0.2 mL serum-free RPMI-1640 media were gently added. After 24 hours incubation at 37°C and 5% CO_2 non-migrated cells on the top surface of membrane were carefully removed. Migrated cells on the bottom surface of the membrane were fixed with methanol and stained with 0.2% crystal violet. Inserts were imaged using Leica DM IL inverted microscope and 200 x total magnification. Number of cells counted is shown as migration level relative to control samples.

3.2.13 Statistical Analyses

GraphPad Prism Software 6.0 was used to generate all graphs and analyses. Statistical analysis was performed using the Mann-Whitney U-test or a two-tailed Student's *t*-test, a threshold of p-value < 0.05 was considered significant. Graphic representation values are presented as mean ± SEM. For correlation analysis, Pearson correlation co-efficient was used for graphical representation of correlation analysis between miRNA and gene expression values. Only correlation with a r of ≤ -0.5 and adjusted p-value ≤ 0.01 was considered significantly negatively correlated. All experiments were repeated three times independently.

3.3 Results

3.3.1 Clinical Information

Clinical characteristics of the patient populations are shown in Table 3.1. There were no differences in maternal age, BMI or gestational age at delivery between patient groups. Each patient group has an equal number of male and female outcome pregnancies. There were significant differences in birth, placental weights and blood pressure between patient groups with complicated pregnancies and gestational age-matched controls. Birth and placental weights were also significantly lower in the PE + IUGR group compared to the PE only and IUGR only groups. These patient populations were the same populations used for the sequencing expression study (Chapter 2). These patients were selected using stringent inclusion and exclusion criteria to include patients with primarily placental factors underlying the diseases. Patients with known maternal and/or fetal risk factors were not included (see methods).

Table 3.1 Clinical characteristics of the patient groups with complicated pregnancies and gestational age-matched controls.

Characteristic (Mean ± SD)	PE N=20	IUGR N=18	PE + IUGR N=20	Control N=21
Maternal Age (years)	28.6 ± 7.0	31.3 ± 5.6	32.6 ± 5.7	28.2 ± 5.0
BMI (kg/m^2)	28.9 ± 7.4	24.5 ± 4.6	28.7 ± 5.3	28.6 ± 7.5
GA at Delivery (weeks)	29.6 ± 3.1	32.3 ± 3.4	29.4 ± 2.5	30.6 ± 2.6
Sex (Females)	10 (50%)	8 (44%)	10 (50%)	11 (52%)
Mode of Delivery: C-Section (%)	15 (75%)	13 (72%)	19 (95%)	4 (19%)
C-Section with Labor (%)	6 (40%)	5 (38%)	5 (26%)	4 (100%)
Birth Weight (grams)	1300 ± 499.7[1]	1256 ± 625.6[2]	933.9 ± 342.2[3]	1803 ± 623.5
Placental Weight (grams)	342.9 ± 135.4[2]	301.5 ± 97.1[3]	244.7 ± 75.2[3,4]	462.7 ± 136.3
Birth Weight Percentile	30.4 ± 19.2	3.7 ± 2.1	4.8 ± 2.0	63.4 ± 26.5
Systolic BP (mm Hg)	173.5 ± 19.1[3,5]	118.6 ± 12.8	170 ± 14.6[3,5]	116.5 ± 15.8
Diastolic BP (mm Hg)	108.0 ± 10.3[3,5]	77.2 ± 12.6	104.3 ± 8.6[3,5]	69.24 ± 13.1
HELLP Syndrome	6 (30%)	NA	8 (40%)	NA

1) p-value < 0.05 vs. control 2) p-value < 0.001 vs. control 3) p-value <0.0001 vs. control 4) p-value <0.01 vs. PE only 5) p-value < 0.0001 vs. IUGR only

Note: Table 3.1 is identical to Table 2.1, the table is provided in this chapter for ease of reference.

3.3.2 Real-time PCR confirmation of candidate genes targets

In our previous study assessing global miRNA expression using NGS in placentae from patients diagnosed with early-onset complications, we identified upregulation of miR-210-5p and miR-193b-5p. miR-210-5p was upregulated in two patient groups (PE, PE +IUGR) , and miR-193b-5p was upregulated in all three patient groups (PE, IUGR, PE +IUGR) compared to gestational age matched controls. These findings were also confirmed using qRT-PCR. Integration of miRNA and gene expression data identified a subset of predicted targets, and the majority of candidate genes were targets for miR-193b-5p and miR-210-5p. Here we confirmed differential downregulation of candidate gene targets for miR-210-5p and miR-193b-5p using qRT-PCR.

Figure 3.1 shows qRT-PCR results for miR-210-5p candidate gene targets in the PE only group, PE + IUGR, or both. All candidate gene targets confirmed downregulation of expression, with the exception of *VAV1* and *WNT3* in the PE only group (Figure 3.1). Figure 3.2 shows qRT-PCR results for miR-193b-5p candidate gene targets. The majority of identified genes are downregulated in the PE + IUGR group (Figure 3.2b). *FGF13* is the only candidate gene target that is down-regulated in all 3 patient groups (Figure 3.2a). All candidate gene targets were confirmed to be downregulated in their respective patient group, with the exception of *CCR1* and *NTN4* in the PE only group (Figure 3.2).

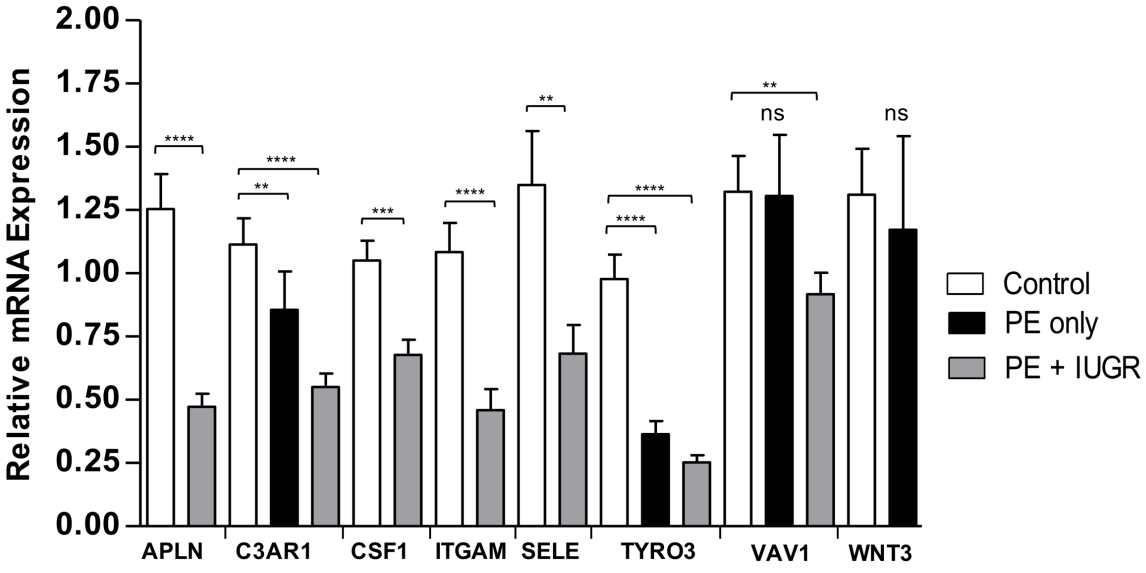

Figure 3.1 mRNA expression levels of candidate gene targets for miR-210-5p

To find relative mRNA expression of candidate gene targets the $\Delta\Delta C_T$ method was used and values were normalized to GAPDH expression. All samples were run in triplicate. Data is shown as the mean ± SEM, significant differences were determined using a Mann-Whitney U test. * p-value <0.05

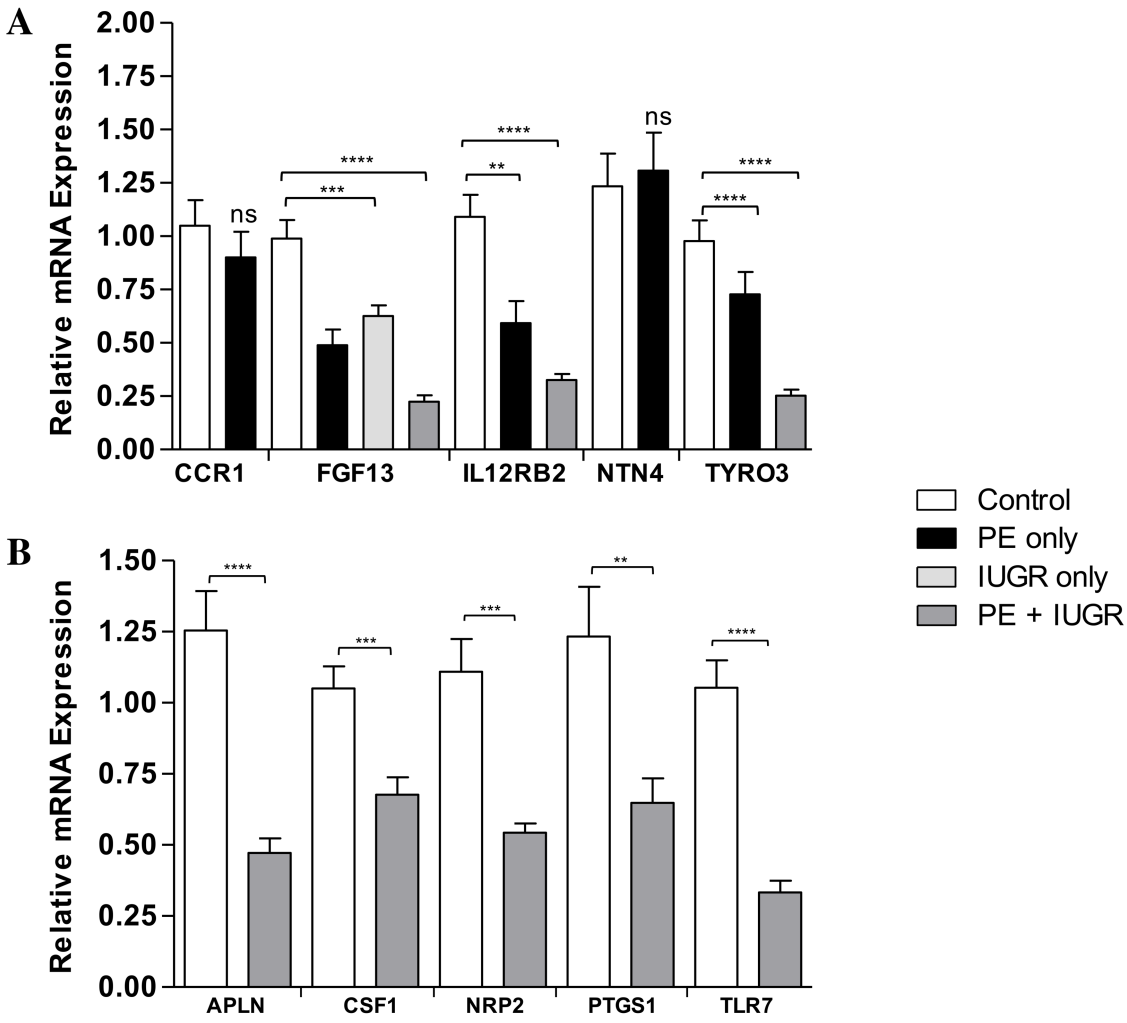

Figure 3.2 mRNA expression levels of candidate gene targets for miR-193b-5p

To find relative mRNA expression of candidate gene targets the $\Delta\Delta C_T$ method was used and values were normalized to GAPDH expression. **A**. Candidate targets identified primarily in more than one patient group. **B.** Candidate targets identified only in patients with PE + IUGR. All samples were run in triplicate. Data is shown as the mean ± SEM, significant differences were determined using Mann-Whitney U test. ** p-value <0.01

3.3.3 Validating candidate gene targets

To confirm the interaction between miRNAs and candidate gene targets, a human trophoblast cell line, HTR-8/SVneo was used for luciferase assays. HTR-8/SVneo cells were co-transfected with plasmids containing the firefly luciferase and the 3′UTR of the target gene of interest, along with miRNA mimics or NC mimics. Plasmids containing the firefly luciferase along with the GAPDH 3′UTR or an empty 3′UTR were used as controls.

3.3.3.1 miR-210-5p targets

Based on qRT-PCR results *VAV1* and *WNT3* were not selected for validation using luciferase assays. We conducted luciferase assays for *C3AR1*, *CSF1*, *ITGAM* and *TYRO3*. Significant decrease in relative luciferase activity was observed in HTR-8/SVneo cells containing 3′UTRs of either *CSF1* or *ITGAM* (Figure 3.3a). However, no changes were observed in cells containing the 3′UTRs of *C3AR1* or *TYRO3* (data not shown). Both *CSF1* and *ITGAM* were predicted targets by more than one software prediction tool at the same nucleotide positions, including TargetScan and miRanda. miR-210-5p is predicted to target *CSF1* at 2380-2387 nt region of the 3′UTR, and *ITGAM* at the 3887-3894 nt region of the 3′UTR (Figure 3.3 b). Inverse correlation analysis using sequencing data had previously shown significant negative inverse correlation between the expression of miR-210-5p and *CSF1* (r = - 0.81), and between miR-210-5p and *ITGAM* (r = - 0.80) in the control and PE + IUGR groups (Figure 3.3 c, d).

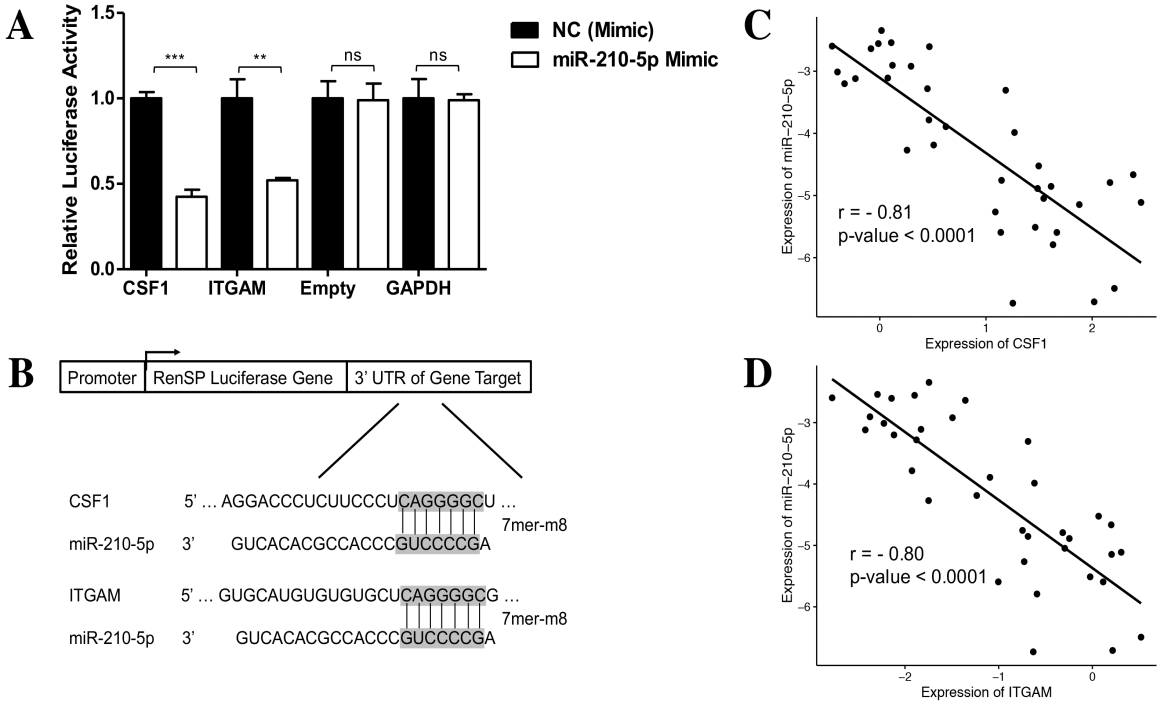

Figure 3.3 Validation of miR-210-5p candidate gene targets

A. Relative luciferase activity measured 24 hours after co-transfection of HTR-8/SVneo cells with luciferase constructs containing 3′UTR of *CSF1*, or *ITGAM*, or control constructs, along with miR-210-5p mimic or NC mimic. Data is shown as mean ± SEM; ** indicates p-value < 0.01 **B.** Schematic of the luciferase construct and sequence alignment between miR-210-5p and gene targets. Significant negative correlation between the expression values of **C.** miR-210-5p and *CSF1* and **D.** miR-210-5p and *ITGAM* obtained from miRNA and RNA-seq expression datasets in patients with PE + IUGR and gestational age-matched controls.

3.3.3.2 miR-193b-5p targets

Based on qRT-PCR results, *CCR1* and *NTN4* were not selected for validation using luciferase assays. Based on assay availability and prior knowledge of candidate gene targets, we prioritized conducting luciferase assays for *APLN*, *CSF1*, *FGF13* and *TYRO3*. Significant decrease in relative luciferase activity was observed in HTR-8/SVneo cells containing 3´UTRs of either *APLN* or *FGF13* (Figure 3.4a). However, no changes were observed in cells containing the 3´UTRs of *CSF1* or *TYRO3* (data not shown). *APLN* and *FGF13* were predicted targets by more than one software prediction tool at the same nucleotide positions. miR-193b-5p is predicted to target *APLN* at 1243-1249 nt region of the 3´UTR, and *FGF13* at the 741-747 nt region of the 3´UTR (Figure 3.4 b). Inverse correlation analysis using sequencing data had previously shown significant negative inverse correlation between the expression of miR-193b-5p and *APLN* (r = - 0.82), and between miR-193b-5p and *FGF13* (r = - 0.82) in the control and PE + IUGR groups (Figure 3.4 c,d). Since *FGF13* is down-regulated in the PE only and the IUGR only groups, there is also significant inverse correlation between the expression of miR-193b-5p and *FGF13* in these groups (r = -0.82 and -0.77 respectively) (Figure 3.4 e,f).

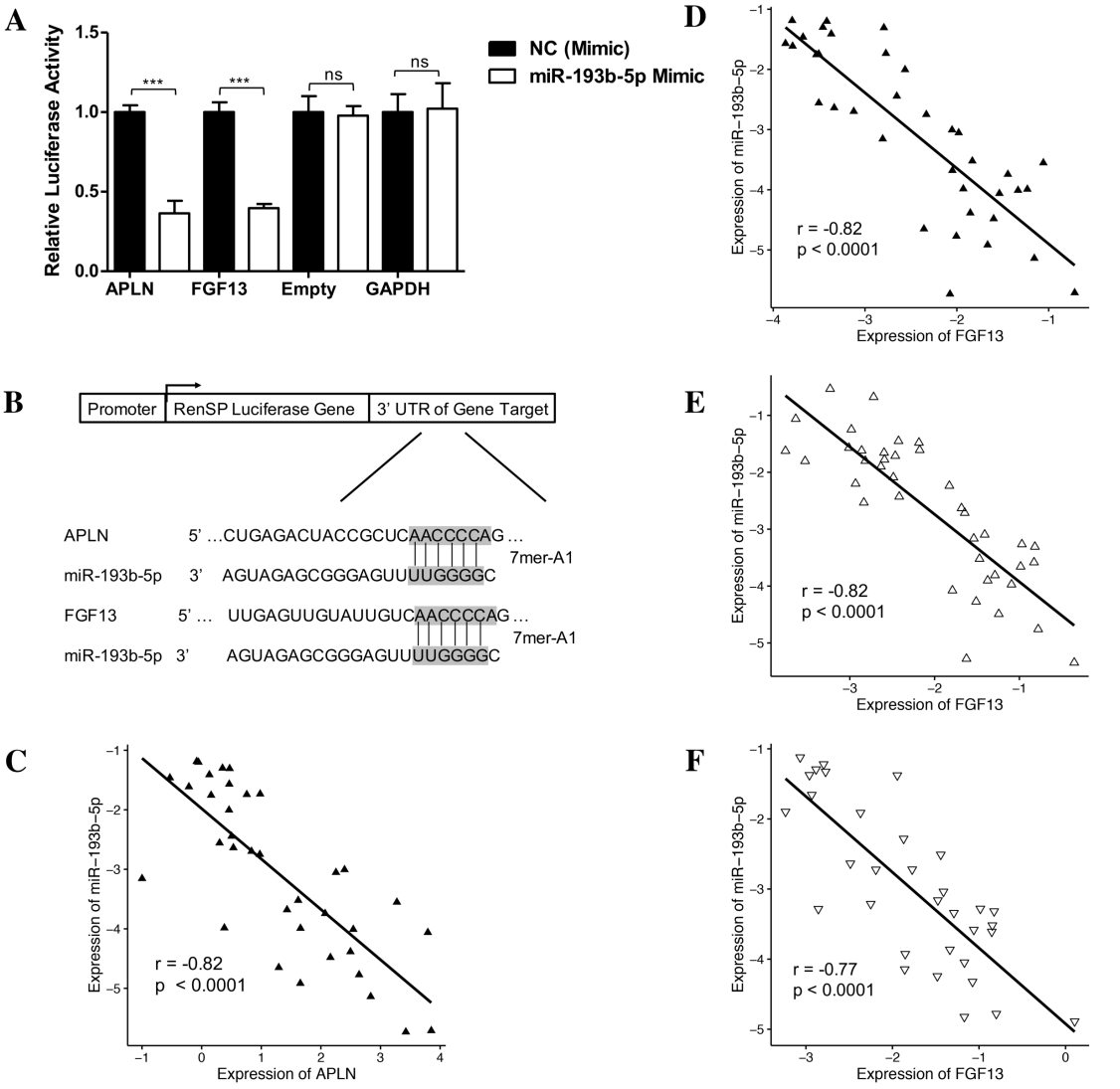

Figure 3.4 Validation of miR-193b-5p candidate gene targets

A. Relative luciferase activity measured 24 hours after co-transfection of HTR-8/SVneo cells with luciferase constructs containing 3′UTR of *APLN*, or *FGF13*, or control constructs, along with miR-193b-5p mimic or NC mimic. Data is shown as mean ± SEM; *** indicates p-value < 0.001 **B.** Schematic of the luciferase construct and sequence alignment between miR-193b-5p and gene targets. Significant negative correlation between the expression values of **C.** miR-193b-5p and *APLN* in the PE + IUGR group,

and miR-193b-5p and *FGF13* in the **D.** PE + IUGR group, **E.** PE only group, and **F.** IUGR only group obtained from miRNA and RNA-seq expression datasets.

3.3.4 Immunohistochemical analysis of gene targets in the placenta

Immunohistochemical analysis of the placenta was conducted to identify which cell types most prominently express gene targets. Staining was completed in PE + IUGR samples (N=2) and gestational-age matched preterm control samples (N=2) for localization of each gene target in both patient groups. For each sample both CV and BPD were stained from whole sections obtained from central and peripheral regions of the placenta. Each gene target evaluated localized to the same cell types in control and PE +IUGR samples.

CSF1 strongly localized to Hofbauer cells in tertiary chorionic villi, meanwhile lighter staining was observed in the CT and SCT cells (Figure 3.5). ITGAM localized to SCT cells in tertiary villi, and both ITGAM and CSF1 were expressed in intermediate CT cells within the basal plate decidua (Figure 3.5). APLN and FGF13 strongly localized to SCT cells, whereas APLN also localized to CT cells and the chorionic villus stroma (Figure 3.6). In the BPD, APLN and FGF13 localized to intermediate CT cells (Figure 3.6).

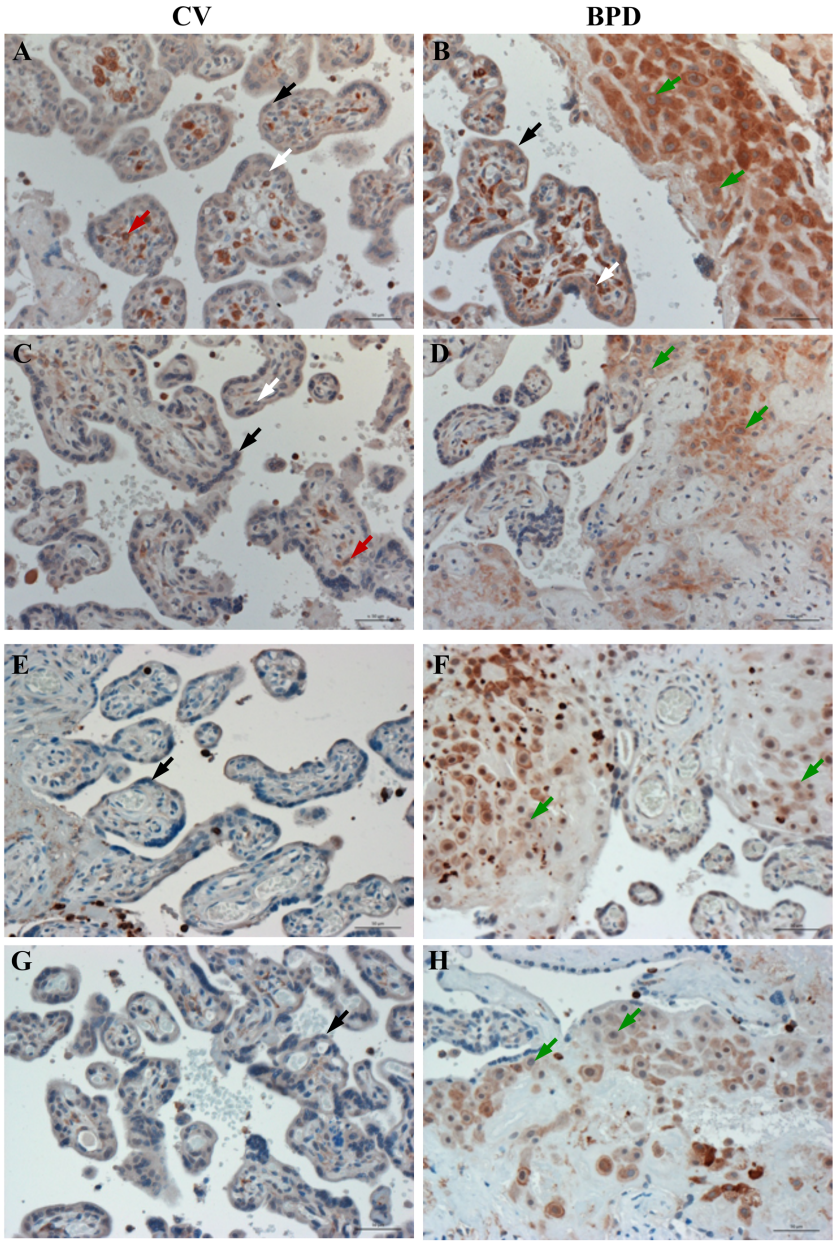

Figure 3.5 Immunohistochemical staining for gene targets CSF1 and ITGAM

Staining for CSF1 in preterm control placenta (**A**,**B**) and in early-onset PE + IUGR (**C**,**D**), gestational age 29+4 and 29+6 respectively. Staining for ITGAM in preterm control placenta (**E**,**F**) and in early-onset PE + IUGR (**G**,**H**), gestational age 31+5 and 32+1 respectively. Black arrows show positivity in SCT cells, white arrows show

positivity in CT cells, red arrows show positivity in Hofbauer cells, green arrows show positivity in intermediate CT cells. All images were captured at 200 x total magnification.

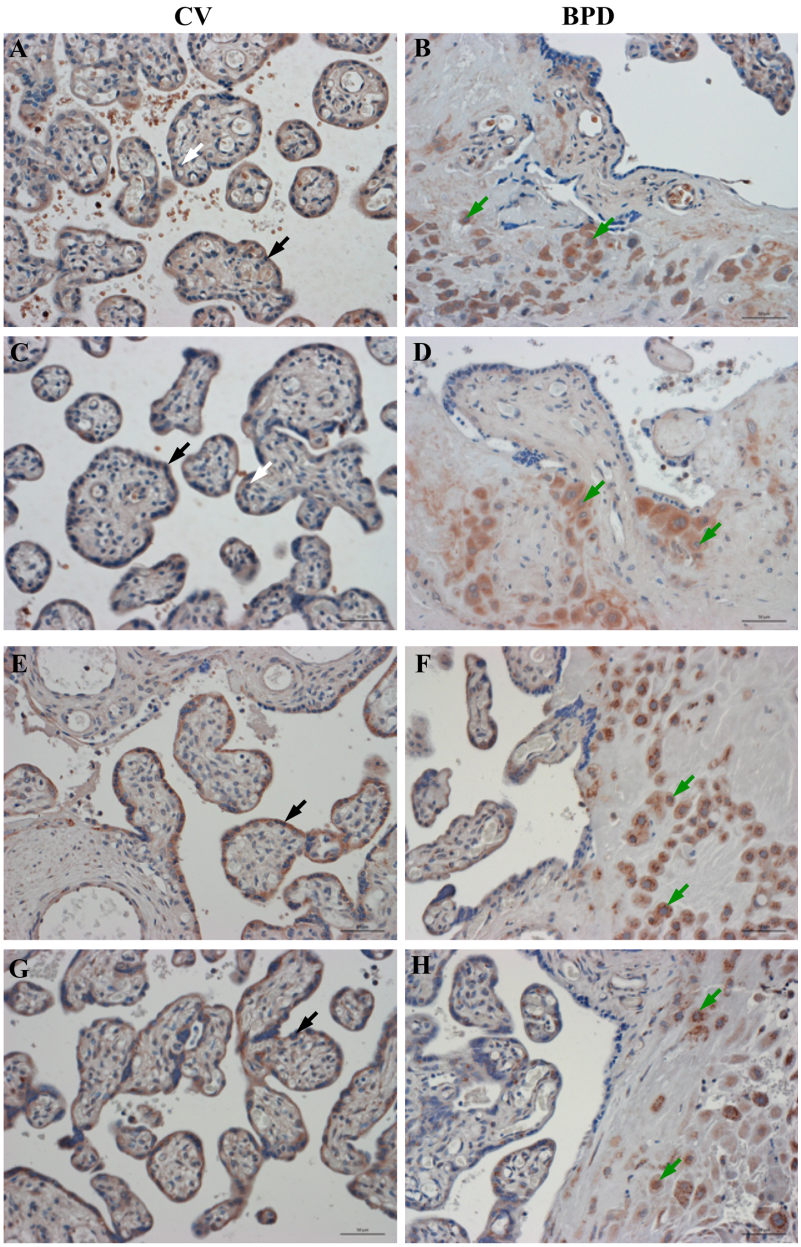

Figure 3.6 Immunohistochemical staining for gene targets APLN and FGF13

Staining for APLN in preterm control placenta (**A**,**B**) and in early-onset PE + IUGR (**C**,**D**), gestational age 29+4 and 29+6 respectively. Staining for FGF13 in preterm

control placenta (**E**,**F**) and in early-onset PE + IUGR (**G**,**H**), gestational age 31+5 and 32+1 respectively. Black arrows show positivity in SCT cell, white arrows show positivity in CT cells, green arrows show positivity in intermediate CT cells. All images were captured at 20 x.

3.3.5 Expression levels of candidate gene targets in HTR-8/SVneo cells

3.3.5.1 miR-210-5p

HTR-8/SVneo cells were transfected with either miR-210-5p mimics or inhibitors. Following treatment, mRNA and protein expression levels of *CSF1* and *ITGAM* were measured in these cells and compared to cells transfected with NC mimics or NC inhibitors. Transfection of miR-210-5p mimics into HTR-8/SVneo cells resulted in a decrease in *CSF1* and *ITGAM* mRNA expression (Figure 3.7 a,b). Conversely, transfection of miR-210-5p inhibitors into HTR-8/SVneo cells increased *CSF1* and *ITGAM* mRNA expression (Figure 3.7 a, b). Similar trends in expression were observed for CSF1 protein after transfection (Figure 3.7 c, d). ITGAM protein in HTR-8/SVneo cells was undetectable using two different commercially available antibodies, although detectable in placental tissues using the same antibodies (data not shown). ITGAM protein was also undetectable in BeWo cells, another trophoblast cell line (data not shown).

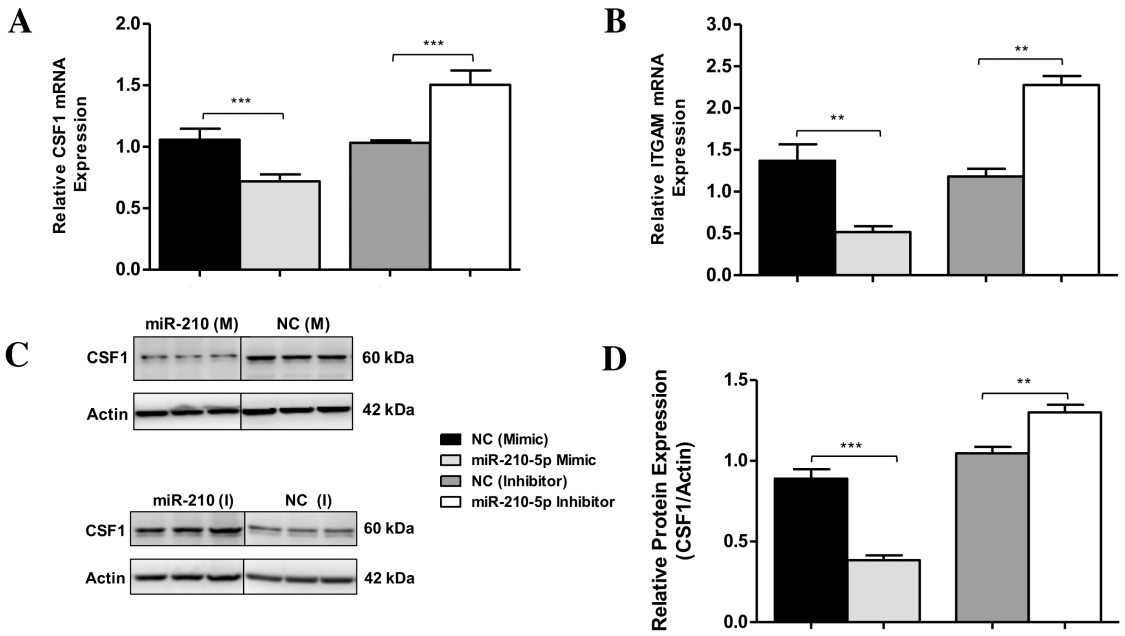

Figure 3.7 Impact of miR-210-5p on target gene expression

A. *CSF1* and **B.** *ITGAM* mRNA expression in HTR-8/SVneo cells transfected with miR-210-5p mimics or inhibitors and compared to the corresponding control (NC mimic or inhibitor) as detected by qRT-PCR and normalized to GAPDH expression using the $\Delta\Delta C_T$ method. **C.** Western blot analysis showed CSF1 protein levels in HTR-8/SVneo cells following transfection with miR-210-5p mimics (top panel) or inhibitors (lower panel) and compared to the corresponding control (NC mimic or inhibitor) **D.** Summary graph from three independent experiments, CSF1 density was normalized to β-actin in the same blot. Values represent mean ± SEM; ** indicates p-value < 0.01

3.3.5.2 miR-193b-5p

HTR-8/SVneo cells were also transfected with either miR-193b-5p mimics or inhibitors. Following treatment, mRNA and protein expression levels of *APLN* and *FGF13* were measured in these cells and compared to cells transfected with NC mimics or NC inhibitors. Transfection of miR-193b-5p mimics into HTR-8/SVneo cells resulted in a decrease in *APLN* and *FGF13* mRNA expression (Figure 3.8 a,b). Meanwhile transfection of miR-193-5p inhibitors into HTR-8/SVneo cells increased *APLN* and *FGF13* mRNA expression (Figure 3.8 a, b). No changes were observed in APLN or FGF13 protein levels after transfection with miR-193b-5p mimics or inhibitors (Figure 3.8 c,d).

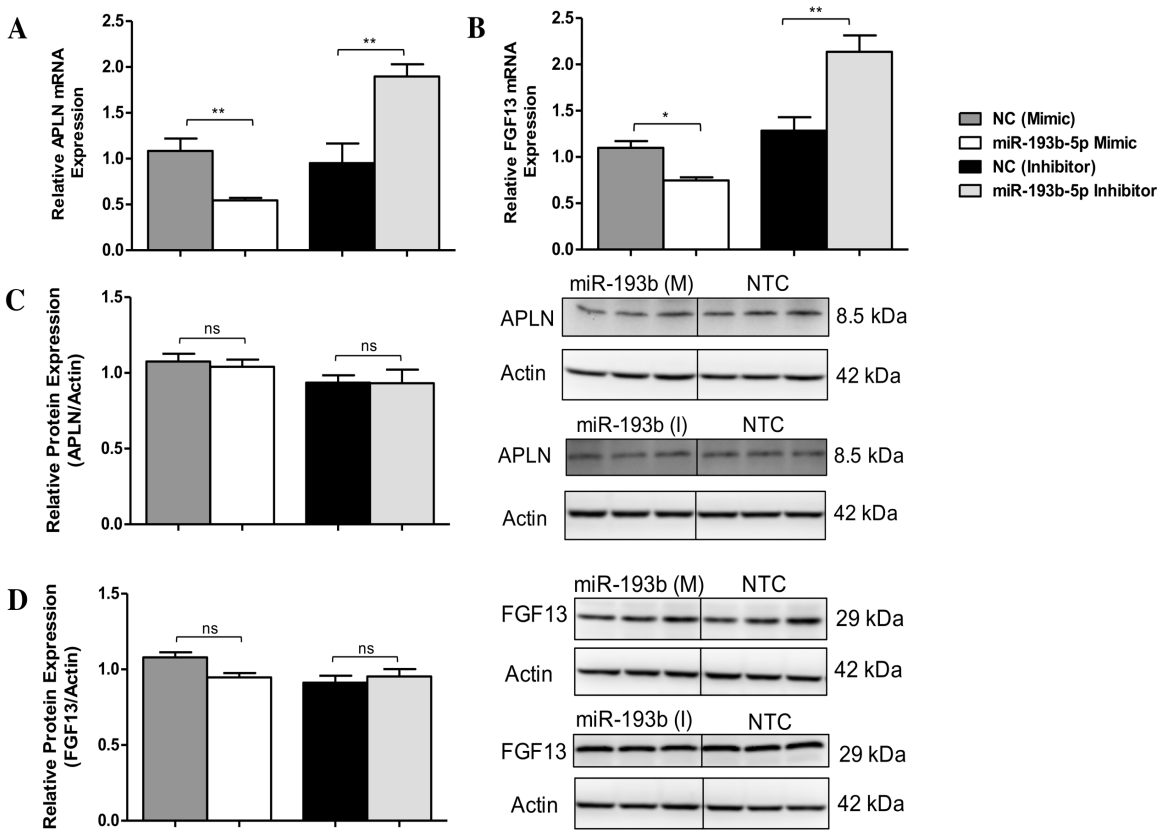

Figure 3.8 Impact of miR-193b-5p on target gene expression

A. *APLN* and **B.** *FGF13* mRNA expression in HTR-8/SVneo cells transfected with miR-193b-5p mimics or inhibitors and compared to the corresponding control (NC mimic or inhibitor) as detected by qRT-PCR and normalized to GAPDH expression using the $\Delta\Delta C_T$ method. Western blot analysis to show **C.** APLN and **D.** FGF13 protein levels in HTR-8/SVneo cells following transfection with miR-193b-5p mimics (top panel) or inhibitors (lower panel) and compared to the corresponding control (NC mimic or inhibitor). Protein density was normalized to β-actin in the same blot. Values represent mean ± SEM; * indicates p-value < 0.05

3.3.6 Impact on trophoblast proliferation and migration

To investigate the impact of miRNAs on cell functions, HTR-8/SVneo cells were transfected with miRNA mimics, inhibitors, or corresponding controls and cell proliferation and migration were assessed. Cell proliferation was measured with a WST-1 reagent following transfections. Cell migration was measured using a wound healing assay and a transwell assay.

3.3.6.1 miR-210-5p

Transfection of HTR-8/SVneo with miR-210-5p mimics decreased proliferation and migration of cells. The fraction of viable cells was 20% less in cells treated with miR-210-5p mimics compared to cells treated with NC mimics. Meanwhile the wound healing assay showed a 30% decrease in relative migration levels, and the transwell assay showed a 60% reduction. Transfection of HTR-8/SVneo with miR-210-5p inhibitors had no impact on proliferation but promoted migration of cells (Figure 3.9). After treatment with miR-210-5p inhibitors the wound healing assay showed a 35% increase in relative migration levels, meanwhile the transwell assay showed a 65% increase.

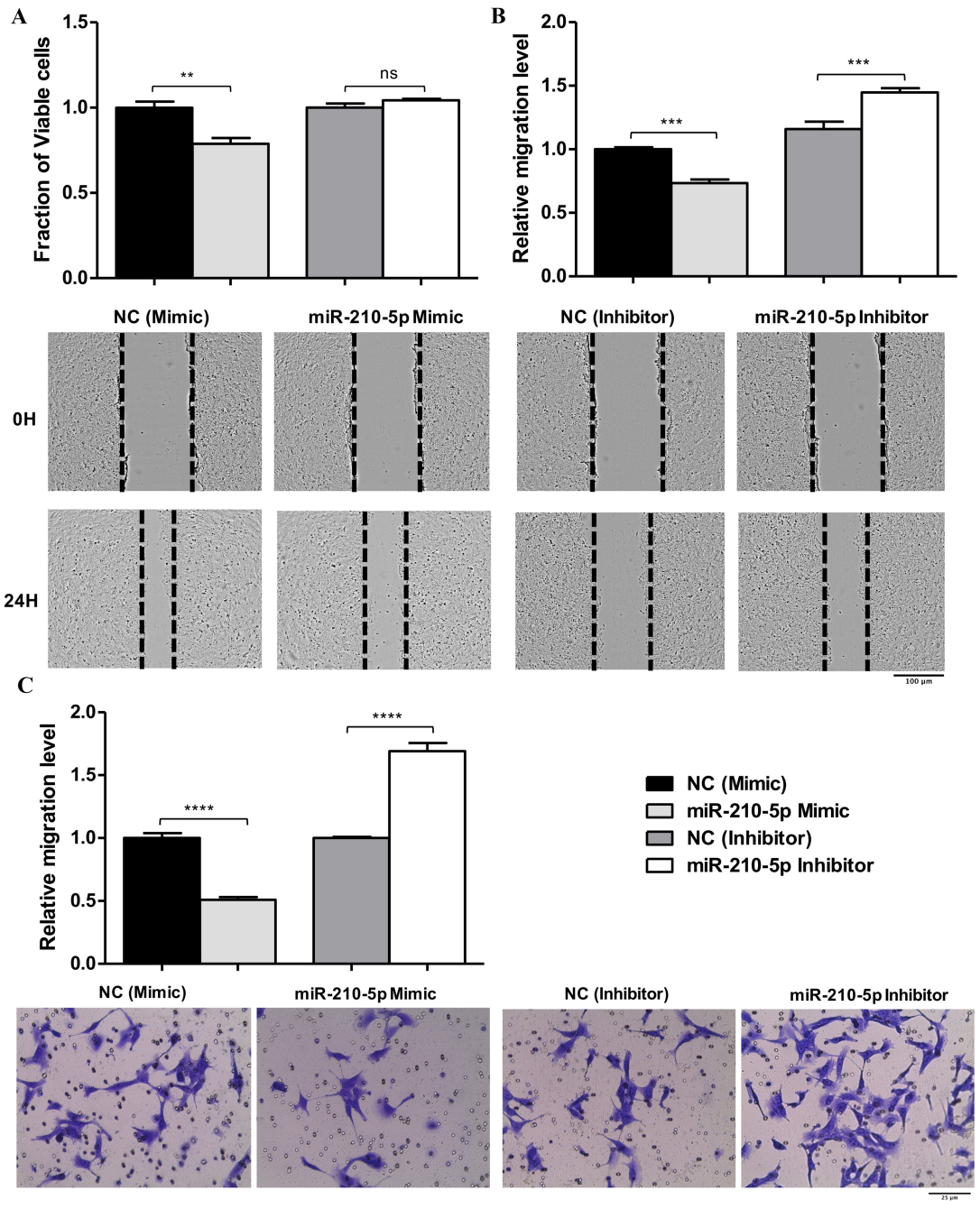

Figure 3.9 miR-210-5p impact on cell functions in human trophoblast cells

A. Effect of miR-210-5p on cell proliferation was investigated using WST-1 reagent. Cells were incubated with WST-1 reagent following transfection with miR-210-5p mimic

or inhibitor and compared to the corresponding control (NC mimic or inhibitor). **B.** To investigate the effect of miR-210-5p overexpression and inhibition on cell migration, HTR-8/SVneo cells were transfected with miR-210-5p mimics, inhibitor, or the corresponding control, scratches were then created and the width of the scratch in each experimental group was measured at time 0 and at 24 hours using 40 x total magnification. Migration level is the distance traveled in 24 hours relative to the control group. **C.** Transfected HTR-8/SVneo cells were transferred into a transwell chamber to assess impact on migration; images were taken 24 hours after seeding using 200 x total magnification. Migration level is the number of cells migrated through the membrane relative to the control group. All experiments were repeated three times independently, data is shown as mean ± SEM ** indicates $P < 0.01$

3.3.6.2 miR-193b-5p

Transfection of HTR-8/SVneo cells with miR-193b-5p mimics decreased proliferation and migration of cells (Figure 3.10). The fraction of viable cells was 25% less in cells treated with miR-193b-5p mimics compared to cells treated with NC mimics (Figure 3.10a). On the other hand, the wound healing assay showed a 35% decrease in relative migration levels, and the transwell assay showed a 50% reduction (Figure 3.10 b, c). Transfection of HTR-8/SVneo with miR-193b-5p inhibitors had no impact on proliferation but promoted migration of cells (Figure 3.10). After treatment with miR-193b-5p inhibitors the wound healing assay showed a 25% increase in relative migration levels, meanwhile the transwell assay showed a 45% increase (Figure 3.10 b, c).

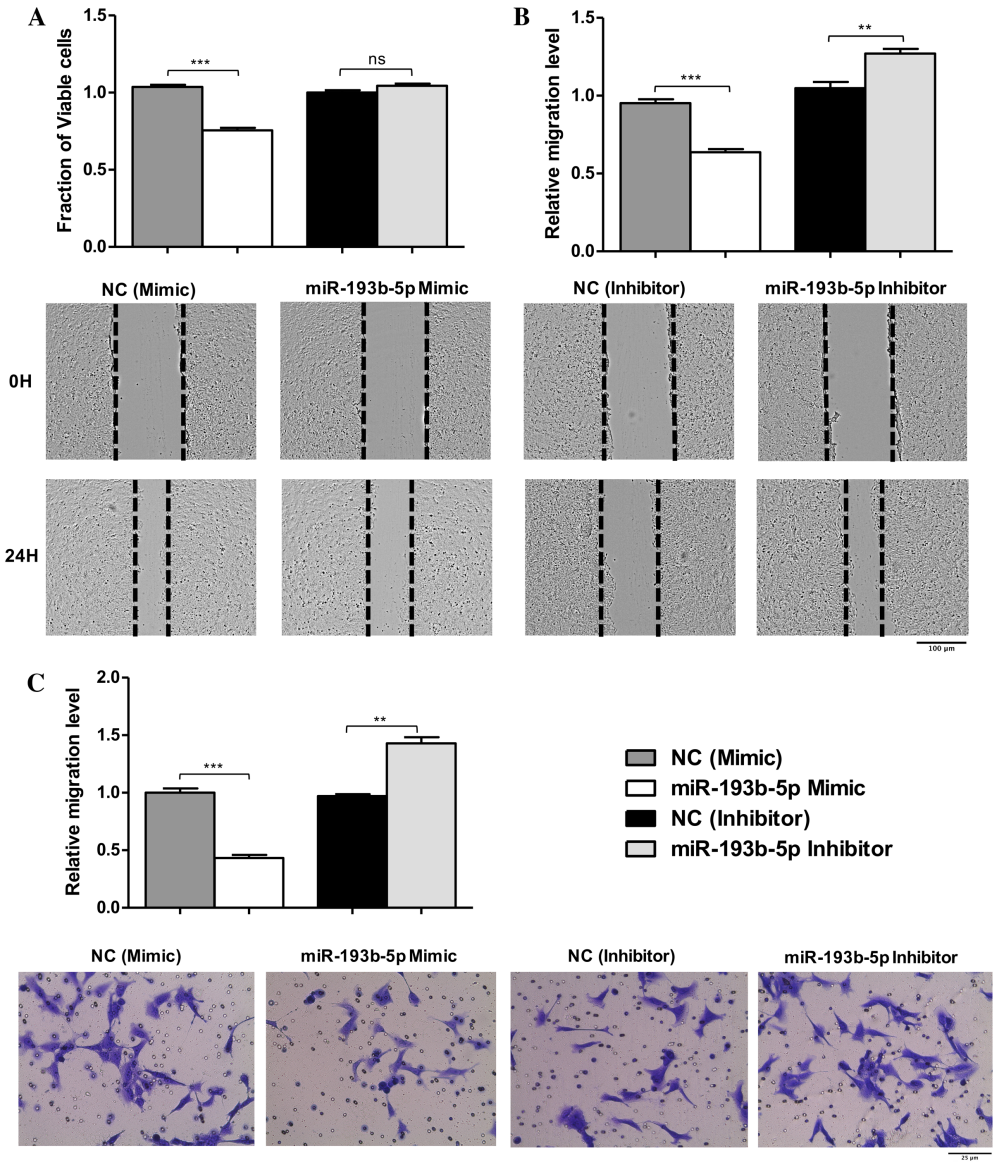

Figure 3.10 miR-193b-5p impact on cell functions in human trophoblast cells

A. Effect of miR-193b-5p on cell proliferation was investigated using WST-1 reagent. Cells were incubated with WST-1 reagent following transfection with miR-193b-5p mimic or inhibitor and compared to the corresponding control (NC mimic or inhibitor). **B.** To investigate the effect of miR-193b-5p overexpression and inhibition on cell migration, HTR-8/SVneo cells were transfected with miR-193b-5p mimics or inhibitor,

or the corresponding control, scratches were then created and the width of the scratch in each experimental group was measured at time 0 and at 24 hours using 40 x total magnification. Migration level is the distance traveled in 24 hours relative to the control group. **C.** Transfected HTR-8/SVneo cells were transferred into a transwell chamber to assess impact on migration, images were taken 24 hours after seeding using 200 x total magnification. Migration level is the number of cells migrated through the membrane relative to the control group. All experiments were repeated three times independently, data is shown as mean ± SEM ** indicates $P < 0.01$

3.4 Discussion

3.4.1 MicroRNAs

In our previous study investigating miRNA expression in placenta from patients diagnosed with early-onset pregnancy complications, we identified significant upregulation of miR-210-5p in patients with PE (± IUGR) and miR-193b-5p in all three pregnancy complicated patient groups (PE only, IUGR only, PE +IUGR). Gene expression data from the same patients allowed us to identify predicted gene targets for differentially expressed miRNAs. A significant number of predicted genes identified were targets for miR-210-5p and/or miR-193b-5p, therefore these two miRNAs became of primary interest.

3.4.1.1 miR-210

miR-210 is one of the most widely identified miRNAs in placentae from complicated pregnancies, it is identified to be upregulated in placenta from patients with PE only, and

patients with PE and SGA babies.[18,22,23] In the human placenta, using *in situ* hybridization, miR-210 expression has been localized to the villous trophoblast and the extravillous interstitial trophoblast.[22] In addition, miR-210 has been widely investigated for its potential use as a diagnostic biomarker, both miR-210-3p and miR-210-5p expression levels were found to be significantly higher in maternal plasma from PE patients.[19,20]

As previously mentioned, the intrauterine environment in pregnancies complicated by PE and/or IUGR can be hypoxic due to decreased perfusion of maternal blood into the uteroplacental unit.[15] It is now known that under hypoxic conditions, miR-210 is upregulated in expression, and upregulation is mediated by the transcription factors HIF-1α or NF-κB.[16,17] Isolated trophoblast cells from first trimester placenta cultured under hypoxic conditions, show significant increases in miR-210 expression.[17] To assess the impact of miR-210 upregulation in PE placenta, studies identified gene targets that are downregulated and are implicated in processes important for placental function.[17,22] Gene targets validated using cell culture methods include: *EFNA3*, *HOXA1*, *ISCU*, *KCMF1*, and *THSD7A*.[17,22,24,25] Validated gene targets implicate miR-210 in important functions such as cell migration, invasion, growth/proliferation, and mitochondrial metabolism.[17,24-27] Based on evidence implicating miR-210-3p in important trophoblast cell functions [24-27] we sought to assess the impact of miR-210-5p on HTR-8/SVneo cell proliferation and migration. Transfection of cells with miR-210-5p mimics reduced proliferation and migration of cells, while inhibition of miR-210-5p only had an effect on cell migration (Figure 3.9).

3.4.1.2 MiR-193b-5p

Previous reports assessing placental miRNA expression in PE pregnancies identified an upregulation in miR-193b-3p and -5p.[28-31] Previous reports including those that utilized high throughput sequencing identified miR-193b-3p and -5p, including our own study.[29,31] Meanwhile studies using array-based technology only identified miR-193b-3p to be upregulated.[28,30,32] To investigate whether miR-193b-5p had impact on cell functions similar to its complementary strand miR-193b-3p, we measured cell proliferation and migration. miR-193b-3p has been shown to target *TGFβ2* in trophoblast cell culture, where the increase in miR-193b-3p expression reduced *TGFβ2* expression and reduced the cell's capacity for migration and invasion.[31] In cancer cells, miR-193b-3p is shown to regulate cell proliferation, migration, and invasion.[33,34] Meanwhile, miR-193b-5p has only been shown to impact cell proliferation in cancer cells.[35] In this study, miR-193b-5p upregulation had pronounced impact on cell migration, shown using a wound healing and transwell assays, and only a modest but significant decrease in cell proliferation (Figure 3.10). Inhibition of miR-193b-5p only had significant effects on cell migration (Figure 3.10).

3.4.2 Trophoblast Cells

To better understand the function and development of trophoblast cells in the placenta, a number of trophoblast cell lines have been created *in vitro*. Freshly isolated CT cells from the placenta can also be used, however these cells do not proliferate in culture and have a short life span *in vitro* (maximum 7 days) where they also fuse to form syncytia.[36,37] The

short life span of freshly isolated CT cells makes molecular manipulation of these cells difficult, therefore immortalized trophoblast cell lines have been created to study trophoblast biology and function.[36,37] As previously mentioned, trophoblast cells in the placenta differentiate into the VT and EVT pathways.[38] Villous CT cells are responsible for the growth and expansion of the placental exchange site, they fuse to form SCT cells which cover the villi, and are important for hormone production, and gas, nutrient and waste exchange.[38] Extravillous CT cells on the other hand, adopt migratory and invasive properties required to invade the maternal myometrium and remodel spiral arteries properly to accommodate for increased demand of blood supply.[38] Based on the working hypothesis that EVT cell dysfunction and improper spiral artery remodeling are potential underlying causes for pregnancy complications such as PE and IUGR, there was and still is strong emphasis on creating *in vitro* systems to study EVT cell properties, particularly how epithelial-to-mesenchymal transition is regulated.[39,40] The mostly commonly used immortalized trophoblast cell line to study EVT cell function is the HTR-8/SVneo cell line. This cell line was created by Graham *et al.* in 1993 by isolating EVT cells from first trimester villous explants from terminated pregnancies, called HTR-8.[41] HTR-8 cells were then immortalized by transfecting the gene encoding the simian virus 40 (SV40) large T antigen using electroporation, creating the HTR-8/SVneo cell line.[41]

Based on immunohistochemical analysis in our placental tissues, we identified expression of gene targets in placental trophoblast cells. More specifically, all four gene targets showed strong reactivity in intermediate cytotrophoblast cells (Figure 3.5 and 3.6). In addition, gene expression analysis for gene targets in unmanipulated HTR-8/SVneo cells

revealed abundant mRNA and protein expression for all gene targets, with the exception of ITGAM protein. Previous literature evidence implicates miRNAs in important CT cell functions important for the EVT pathway including proliferation, migration and invasion.[42-44] Finally, global miRNA expression in HTR-8/SVneo cells showed wide expression of miRNAs including placenta-specific miRNAs.[45]

For the study of VT biology, naturally occurring immortalized trophoblast cells from choriocarcinomas are more commonly used. Examples of these cell lines include: BeWo, JEG-3 and JAR.[39,40] Addition of bromo-cAMP to BeWo cell culture triggers fusion and syncytialization of cells.[46] Therefore, the BeWo cell line is commonly used to investigate processes of involving transcellular transport and syncytialization.[46]

3.4.3 Gene Targets

Target prediction tools identified a number of candidate gene targets for miR-210-5p and miR-193b-5p. Luciferase assays were used to test for miRNA : mRNA interactions, and transfection of HTR-8/SVneo cells with miRNA mimics and a luciferase plasmid containing the 3′UTR of the target of interest identified *CSF1* and *ITGAM* as primary targets for miR-210-5p, and *APLN* and *FGF13* as primary targets for miR-193b-5p (Figures 3.3 and 3.4).

Transfection of HTR-8/SVneo with miRNA mimics and inhibitors impacted gene target expression. In cells transfected with miR-210-5p mimics, *CSF1* and *ITGAM* mRNA expression was decreased, whereas protein levels of only CSF1 were decreased (Figure 3.7). ITGAM protein was not detectable in HTR-8/SVneo cells although strongly

expressed in chorionic villi homogenates. Therefore, it is possible that *ITGAM* mRNA translation into a full functional protein may be dependent upon the environment. Culturing trophoblast cells in the presence of other placental cell types such as endothelial cells may trigger mRNA translation into protein.

Transfection of HTR-8/SVneo cells with miR-193b-5p mimics decreased *APLN* and *FGF13* mRNA expression, miR-193b-5p inhibitors increased *APLN* and *FGF13* mRNA expression (Figure 3.8). However, changes in mRNA levels were not reflected in protein levels of APLN and FGF13 with either treatment (Figure 3.8). This can potentially be attributed to (i) presence of multiple transcripts for the same gene, (ii) post-transcriptional modifications, or (iii) limitation of using western blotting as a detection method to measure protein levels.

3.4.3.1 CSF1

In our search for targets, it was interesting to note that CSF1 receptor (CSF1R) was also a predicted target of miR-210-5p. Colony-stimulating factor-1 is a growth factor that is known to regulate proliferation, migration, and differentiation of mononuclear phagocytes, through a transmembrane tyrosine kinase receptor, CSF1R.[47] Expression of CSF1 and its receptor CSF1R has been detected in the placenta.[48-51] Immunohistochemical staining shows widespread CSF1R expression in placental trophoblast in first trimester placenta, meanwhile CSF1 expression was detected in the CT lining the villous core.[50] In placenta from early- and late-onset PE compared to term controls CSF1 was localized to CT and SCT cells, however when quantified no

differences were found between groups.[51] In placental samples from our patient groups CSF1 is localized to SCT and CT layers but is primarily expressed in Hofbauer cells in the CV, and in intermediate CT cells shown in the BPD (Figure 3.5 a-d).

EVT cells propagated in cell culture continue to express CSF1 and CSF1R mRNA and protein, and addition of exogenous CSF1 to EVT cell cultures significantly stimulated proliferation but had no impact on cell invasiveness.[52] Another study suggested a role for CSF1 in trophoblast cell proliferation and showed CSF1 could be acting in part through HLX1 to regulate cell proliferation.[53] In addition, treatment of term placental CTs with exogenous CSF1 in culture, increases the number and size of multinucleated structures forming extended stretches of syncytium, thereby implicating CSF1 in syncytialization of trophoblast cells.[54,55] There is evidence to support that CSF1 can be regulated by miRNAs and is shown in ovarian cancer cells, where CSF1 is a target for miR-128 and miR-152, and overexpression of miRNAs correlated with a decrease in CSF1 expression impacting cell migration and adhesion.[56]

Reports on the expression of macrophage-CSF (M-CSF) and granulocyte-macrophage-CSF (GM-CSF) in blood and placenta from PE pregnancies are conflicting.[51, 57-59] While some studies report an increase in M-CSF levels in the maternal sera and an increase in GM/M-CSF in the placenta, others report no change.[51,57-59] Meanwhile a study in patients diagnosed with IUGR, found M-CSF levels to be significantly lower in amniotic fluid samples.[60] Conflicting reports can be attributed to lack of standardization of the patient selection process, for example grouping early- and late-onset PE patients together or grouping patients with PE ± IUGR together.

3.4.3.2 ITGAM

Integrin subunit alpha M, also known as ITGAM or CD11b, binds non-covalently to a β_2 subunit (CD18) to form integrin $\alpha_M\beta_2$, that is expressed in monocytes, granulocytes, and macrophages.[61,62] CD11b/CD18 have the capacity to recognize a number of ligands, such as fibrinogen, complement fragment iC3b and ICAM-1 to mediate leukocyte adhesion and migration, and are thus implicated in inflammation.[61] Studies have shown the independent role of CD11b and CD18. Cells expressing only the α_M subunit (ITGAM) can recognize ligands, normally recognized by the integrin $\alpha_M\beta_2$, independently of the β_2 subunit, and subsequently mediate firm cell adhesion and spreading in response to these ligands.[61]

Studies measuring CD11b expression in maternal sera or macrophages of the placenta also have conflicting reports.[63-65] In maternal sera from PE patients, one study reports an increase while another reports no change.[63,64] In placental macrophages, two studies isolated Hofbauer cells and only one reported ITGAM expression.[65,66] Meanwhile, in trophoblast cell culture, a study investigating the role of chemokines in promoting trophoblast cell migration via regulation of adhesion molecules and extracellular matrix components, ITGAM showed a two-fold increase in trophoblast cells upon treatment with chemokines.[67] A more recent study utilized a microarray approach for transcriptional profiling of the syncytiotrophoblast, invasive, and endovascular cytotrophoblast layers in placenta from women with severe PE, using preterm births as controls.[68] RNA profiles show increased expression of ITGAM in the endovascular cytotrophoblast samples compared to the syncytiotrophoblast and invasive cytotrophoblast samples from both PE

and preterm placenta.[68] However, there were no differences in ITGAM expression in any of the samples between PE and preterm placenta.[68] In pregnant mice, the expression of ITGAM has been strongly localized to the spongiotrophoblast layer and was shown to increase across gestation.[69] In this study staining for ITGAM in whole placental tissues localized ITGAM to intermediate CT cells in the BPD (Figure 3.5 e,f).

3.4.3.3 APLN

Apelin is the ligand for the G-protein-coupled receptor angiotensin II receptor-like 1, also known as APJ.[59,60] The role of APLN is well investigated in the cardiovascular system and is known to play a role in angiogenesis.[70-72] The exact role of APLN in the human placenta is not yet fully elucidated, however both APLN and APJ are expressed in the human placenta, with evidence of temporal expression, decreasing as the placenta matures.[73] More specifically, immunohistochemical staining shows expression of APLN and APJ in the CT, SCT and vascular endothelial cells of the placenta.[73-75] In this study staining in preterm controls and PE +IUGR placenta localizes APLN to the same cell types as well as to intermediate cytotrophoblasts in the BPD (Figure 3.6 a-d). As for the expression of APLN in placenta from PE and IUGR pregnancies, while there are some studies that report no differences, many report a downregulation of APLN and APJ mRNA and protein in severe PE patients.[73-77] A severe down regulation of APLN is noted particularly in patients with PE and IUGR.[73,77] In our previous report of gene expression profiling in patients with early onset complications, we identified APLN to be only downregulated in patients with PE + IUGR, but not in PE only or the IUGR only groups compared to gestational age-matched controls. Apelin was also recently investigated as a

potential therapeutic agent in a rat model of PE, and was found to inhibit oxidative stress, one of the common hallmarks of PE.[78]

3.4.3.4 FGF13

Fibroblast growth factor-13 (FGF13) belongs to the FGF homologous factor (FHF) subfamily.[79] Unlike most fibroblast growth factors, FGF13 acts intracellularly in a FGFR-independent manner.[79] This intracellular protein is found to be expressed in the nervous system, including the brain, as well as muscle cells, and more recently in cancer cells.[80-82] In the nervous system FGF13 is an important regulator of neuronal development, as it plays a role in neural differentiation and regulates the migration and polarization of neurons.[80,83] In cancer cells, FGF13 is upregulated, and its upregulation is thought to be beneficial for cancer cells.[82] In human cancer cell lines, knock-down of FGF13 induced apoptosis, whereas overexpression restricted accumulation of reactive oxygen species and promoted cell survival.[82] Although not well investigated in the human placenta, FGF13 is expressed in placenta trophoblasts (results from the Human Protein Atlas Database, http://www.proteinatlas.org/ENSG00000129682-FGF13/tissue). A more recent 2018 report by Yue et al.,[84] identified FGF13 to be expressed in trophoblast cells of the placenta using immunofluorescence and cytokeratin-7 as a positive trophoblast marker.[84] In the same report, FGF13 mRNA and protein are reported to be downregulated in patients with severe PE and is proposed to play a role in trophoblast permeability.[84] We previously reported the down-regulation of FGF13 and using qRT-PCR in this report, we confirmed the down-regulation of FGF13 in patients with early-onset PE only, IUGR only, and PE + IUGR. Immunohistochemical staining

localized FGF13 to SCT cells in the CV and was also localized to intermediate CT cells in the BPD (Figure 3.6 e-h). To our knowledge, this study is the second to report FGF13 down-regulation in early-onset PE patients, and the first to report it in early-onset IUGR only, and PE + IUGR patients.

3.5 Conclusion

In conclusion, this study contributes to our knowledge of miRNAs and their potential impact on placental growth/development and function. Here, we report the upregulation of miR-210-5p and miR-193b-5p in patients with early-onset complications. Luciferase assay results and mRNA expression levels of candidate targets in treated HTR-8/SVneo cells supports potential regulation of CSF1 and ITGAM by miR-210-5p, and APLN and FGF13 by miR-193b-5p. These gene targets were localized to intermediate CT cells in the BPD and potentially play an important role in placental function. Additionally, the two miRNAs impact the ability of HTR-8/SVneo cells to proliferate and migrate, providing further evidence for the role of miRNAs in cellular functions. To further confirm findings, future experiments can focus on: (i) creating point mutations in the 3′UTR regions of gene targets to confirm sequence specificity, (ii) conducting the same experiments in another trophoblast cell line more representative of 3rd trimester placenta, such as BeWo, (iii) use siRNAs to knock down or decrease gene target expression in cell culture and measure functions such as proliferation, migration, and adhesion. Further studies investigating and validating miRNAs and gene-target interactions will be useful in expanding our knowledge of the role of microRNAs in disease, and particularly their impact on trophoblast cells necessary for placental health and function.

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Chapter 4

4 General Discussion

4.1 Summary and Perspective

MiRNAs are short, single-stranded, endogenous RNA molecules that are abundantly expressed in most human tissues. MiRNAs regulate gene expression by targeting mRNA transcripts in the cytoplasm via sequence complementarity. MiRNA targeting mechanisms may then result in either (i) degradation of the mRNA transcript, or (ii) blocking of translational machinery. Some miRNAs are tissue specific, such as miRNA clusters C14MC and C19MC expressed on chromosomes 14 and 19 respectively, in the human placenta.[1] Expression levels of placenta-specific miRNAs are also found to be different in first versus third trimester placenta.[2] Collectively, these discoveries sparked interest in the role of miRNAs in placental growth and function, and particularly their role in disease.

This study utilized NGS technology to measure global expression levels of miRNAs in placental samples from three distinct patient groups diagnosed with early-onset pregnancy complications (PE only, IUGR only, or PE + IUGR) in comparison to placentae from gestational-age matched controls. This not only allowed us to identify miRNAs that are differentially expressed in each disease group, but also to compare the diseased groups to identify miRNAs that are common and unique to each disease. To investigate the potential impact of alterations in miRNA expression on gene expression in the placenta during disease, we again used NGS technology to measure global gene

expression levels in the same patient samples. This analysis also allowed us to identify genes differentially expressed in each disease group as well as genes that are common between the pregnancy complications. Findings from the miRNA and gene expression analyses are reported in Chapter 2.

After completion of the differential expression analysis for miRNAs and genes in the same patient samples, the expression data sets were integrated using inverse correlation analysis. We sought to identify any differentially expressed miRNAs that were significantly inversely correlated with any differentially expressed genes in each patient group, i.e. upregulated miRNA and downregulated gene or *vice versa*. This analysis was used to guide our initial search for potential gene targets impacted by dysregulated miRNAs. The list of potential gene targets of interest was then further refined to include genes that were predicted by software prediction tools for the miRNA of interest. Two miRNAs became of interest following inverse correlation analysis and target prediction, miR-210 and miR-193b. These two miRNAs were upregulated in all three patient groups (PE only, IUGR only, and PE + IUGR) compared to controls, and the majority of predicted genes are targets for either miR-210-5p, miR-193b-5p or in some cases both. Predicted gene targets were identified in all three patient groups, however majority of the genes were in the PE + IUGR patient group.

Chapters 3 focused on investigating the identified candidate gene targets from chapter 2 for miR-210-5p and miR-193b-5p. For each miRNA the studies in chapters 3 aimed to confirm miRNA: mRNA interactions using luciferase assays and to measure impact of miRNA dysregulation on important cell functions such as migration.

4.1.1 MicroRNA expression in early pregnancy complications

MicroRNA differential expression analysis identified six upregulated miRNAs that are common to all three patient groups with early-onset pregnancy complications compared to gestational-age matched controls. All six miRNAs have previously been identified to be upregulated in placenta from PE pregnancies.[3-11] However, none of the six miRNAs have previously been identified in placenta from IUGR pregnancies. Findings from our study overlapped significantly with findings from two other studies, Ishibashi et al. and Zhou et al., that also used NGS technology to measure miRNA expression.[3,7] However, it is important to note that patients used in these two studies are likely diagnosed with late-onset PE.[3,7] Segregation of patient groups into early- and late- onset in scientific studies is beneficial to expand our understanding of molecular mechanisms that are unique based on onset of clinical symptoms, and mechanisms that are common regardless of the gestational age of the onset of symptoms. The most commonly identified dysregulated miRNAs were miR-210 and miR-193b-3p.[3-11] Since miR-210 is one of the first miRNAs to be associated with PE[9], subsequent studies focus on the impact of miR-210 on placental growth and function.[12-15] Indeed, many studies now solely investigate the impact of miRNAs by identifying candidate gene targets and measuring important cell functions, particularly in trophoblast cells.

In our study, a subset of miRNAs remained unique to either PE or IUGR placenta, with the majority of miRNAs in the IUGR only group. Three miRNAs were unique to PE placenta (+/- IUGR) and two more were unique to patients with PE only. The majority of miRNAs differentially expressed in the IUGR only group remained unique to this patient

group (31/37). Studies measuring miRNA expression in placenta from IUGR pregnancies are scarce and overlap between our findings in this patient group compared to other studies is limited. This can be attributed to (i) heterogeneity of disease etiology, (ii) distinction between pathological vs. non-pathological forms of the condition (IUGR vs. SGA), and (iii) differences in patient population (Late- and early-onset, +/- PE). However, there is literature evidence for dysregulation of miRNAs in the C19MC in IUGR pregnancies.[16,17] Our study identified 4 miRNAs that belong to the C19MC cluster and 6 that belong to the C14MC cluster that are dysregulated in IUGR only placenta, emphasizing the potential role of these placenta-specific miRNA clusters in pregnancy complications.[1]

Although pregnancy complications such as PE and IUGR are treated distinctly and have different clinical manifestations, miRNA expression results provide evidence for some common molecular mechanisms in the placenta potentially involved in disease pathogenesis. Although impact on gene expression may vary between disease groups, the underlying mechanisms impacting changes in gene expression maybe similar and directly involved in disease process of PE and IUGR.

4.1.2 Gene expression in early pregnancy complications

Global gene expression analysis in the three patient cohorts revealed results similar to results from the miRNA expression analysis, in that a subset of genes is common to all three patient groups, whereas other genes exhibited specificity to the disease (PE or IUGR) or the specific patient group. A total of 22 genes are differentially expressed in all

three patient groups, many of which have been identified in studies measuring global gene expression in placenta from PE pregnancies (*CST6*, *FSTL3*, *INHA*, *SLCO2A1*, *TGFβ1*).[18-22] To our knowledge only 3 of the 22 genes common to all three patients groups have been reported in multiple disease groups in the literature. One gene is *FSTL3,* which has been identified to be upregulated in placenta from IUGR pregnancies.[25] As well as, *F13A1* and *FAM101B*, which have been reported to be downregulated in a study measuring global gene expression in patients with late-onset PE co-occurring with IUGR, providing evidence for overlapping patterns of gene expression between PE and IUGR.[19] This was also shown more recently in a study conducted by Gibbs et al., where unsupervised cluster analysis of placental transcriptional and histological data from normotensive and hypertensive patients with IUGR revealed overlap between the patient groups based on both transcriptional and histological data.[26]

Differential gene expression analysis also revealed 141 genes unique to patients with PE (+/- IUGR) providing evidence for the large changes in the transcriptome observed in placenta from PE pregnancies. A large subset of these genes overlap with findings in the literature, with both late-[18-21] and early-onset[22] PE studies, but more so in early-onset studies. This emphasizes the importance of segregating patient populations based on onset of symptoms allowing us to compare findings to better understand the underlying molecular manifestations of each form of disease. Studies measuring expression in early- and late-onset PE including this study agree on a core group of genes that are consistently identified to be dysregulated in PE placenta, these genes include but are not limited to: *ENG, FTL1, FSTL3, INHA, INHBA, LEP, HTRA4, SASH1*.[18-22] Global gene expression

studies in placenta from IUGR pregnancies are scarce and overlap with existing studies is limited. This is likely due different definitions of patient populations in these studies that may include both pathological and non-pathological forms of the disease.[27,28] A few genes that did overlap between our patient groups with IUGR and reports in the literature include: *BMP1* in the IUGR only group, *NFASC*, *FAM20A* and *RBP1* in the PE + IUGR group.[23,24] On the other hand, in comparing these studies investigating placental gene expression in IUGR pregnancies it became apparent that many genes within the same family are commonly dysregulated but not the same exact gene, examples include: *ADAM, GFOD, GLRX, NUDT, PLCB, RNF,* and *SCARB*.[23,24]

Findings from this global gene expression study provide further evidence that although PE and IUGR are distinct pregnancy complications, there are common perturbations in placental transcription potentially contributing to both conditions. Meanwhile genes unique to each pregnancy complication provide evidence that there are perhaps maternal and fetal contributions that are observed in PE and IUGR respectively.

4.1.3 MicroRNAs and genes in placental growth/development and function

Investigation of the role of miRNAs in pregnancy complications is an important step to understand the impact of miRNAs on placental health and function. To accomplish this, studies focus on identifying (i) important candidate gene targets for miRNAs and (ii) measure changes in biological and cellular functions. In the last 5 years, there has been a surge in studies investigating the functional role of miRNAs in pregnancy complications

and in other diseases as well, particularly cancer. Table 4.1 shows some of the miRNAs that we report to be differentially expressed in our patient groups and reports in the literature that involve these miRNAs in biological functions important for placental growth and function. Migration, invasion, and proliferation are the most common biological functions associated with these miRNAs. These biological functions are most important in the process of spiral artery remodeling, thought to be dysregulated in pregnancy complications such as PE and IUGR. Intermediate CT cells must differentiate into EVT cells that have migration and invasion capabilities to properly remodel spiral arteries. Studies listed in table 4.1 provide evidence for how miRNAs can be involved in regulating these important biological functions. Studies on miR-210 provide the earliest evidence of the involvement of miRNAs in these biological functions particularly migration and invasion.[12-15] Multiple validated miR-210 targets are known to be involved in regulating EVT cell invasion, they include *EFNA3*, *HOXA9*, and *THSD7A*.[12,14] While miR-210 is upregulated in PE pregnancies, these gene targets are found to be downregulated. Another example is miR-515-5p, a member of C19MC that is upregulated in IUGR placenta and has been validated to target *CYP19A1*, *FZD5*, and *GCM1* three genes important for human trophoblast differentiation.[44] MiRNAs identified in this study and other studies are synthesized in the chorionic villi and their potential role in the regulation of expression of genes involved in the proliferation and invasion of EVTs and remodeling of spiral arteries occurs in the decidual plate of the placenta and the endometrium of maternal uterus. Most miRNAs are synthesized in trophoblast cells and can exert their biological functions in the same cell or nearby cells. It is possible that

the identified miRNAs are expressed in EVTs or nearby cells, however, this was not investigated in this study.

Table 4.1 includes studies reporting on these miRNAs in the context of pregnancy (reference indicated in bold) and in the context of cancer. There are many parallels between placental and tumor development, including mechanisms for regulating proliferation, invasion and immune tolerance.[48-50] Table 4.1 does not represent a comprehensive list of miRNAs, there are more miRNAs identified in the literature showing similar implications in migration, invasion, and proliferation. In addition to identified miRNAs providing evidence for trophoblast cell dysfunction in these pregnancy complications, a subset of genes that this study reported to be differentially expressed in all three patient groups are also directly implicated in the same biological cell functions. Table 4.2 lists 7 genes that are a part of the 22 genes common in patients with PE and/or IUGR that have been shown in the literature to be involved in biological functions such as: migration, invasion, and differentiation. Studies referenced in table 4.2 all directly address the role of these genes in the context of trophoblast cells and direct impact on placental growth and function. In conclusion, both miRNAs and genes commonly dysregulated in all three patient groups provide evidence for trophoblast dysfunction present in placenta from PE and IUGR pregnancies and could be a direct contributor to the co-occurrence of these complications in a subset of patients (PE + IUGR).

Table 4.1 MicroRNAs identified in patient groups also previously described in the literature to impact important biological functions

MicroRNA	Chromosomal Location	Disease Group (current study)	Biological Function	Gene Targets	Reference
miR-210	11p15.5	PE, IUGR, PE + IUGR	Invasion, Migration	*KCMF1, THSD7A, ISCU, EFNA3, HOXA9*	**12**, **13**, **14**, **15**
miR-193b	16p13.12	PE, IUGR, PE + IUGR	Migration, Invasion, Proliferation	*DDAH1, TGFβ2, CCND1, PLK1*	**3**, 29, 30, 31
miR-520a-3p	19q13.42	PE, IUGR, PE + IUGR	Migration, Apoptosis	*EGFR, PARP1*	32, **33**
miR-365a/b-3p	16p13.12/ 17p11.2	PE, IUGR, PE + IUGR	Apoptosis	*SGK1*	**34**
miR-193a-3p	17p11.2	IUGR	Proliferation, Invasion	*ERBB4, MCL1*	35, 36
miR-520f-5p	19q13.42	IUGR	Invasion, Migration, Proliferation	*FGF16, TM4SF1*	37, 38
miR-101	1p31.3	IUGR	Apoptosis, Invasion	*ERp44, SPRY4*	**39**, 40
miR-154-3p	14q32.31	IUGR	Migration, Invasion, Proliferation	*HMGA2, CCND2, TLR2*	41, 42, 43
miR-515-5p	19q13.42	IUGR	Differentiation, Migration	*CYP19A1 FZD5 GCM1 MARK4*	**44**, 45
miR-141-3p	12p13.31	IUGR	Proliferation, Invasion, Intracellular Communication	*LIF*	46, **47**

References in **bold** indicate studies measuring impact on biological function within the context of the placenta and/or pregnancy complications.

Table 4.2 Genes identified in all patient groups with pregnancy complications are important for trophoblast biological functions

Gene	Biological Function (In Placental Trophoblast cells)	Reference
CST6	Differentiation	51, 52
FGF13	Permeability	53
FSTL3	Differentiation, Migration, Invasion, Proliferation	54, 55
HTRA1	Migration, Invasion	56, 57, 58, 59
INHA	Differentiation, Syncytial Fusion	51, 60, 61
ITGA5	Adhesion	62, 63
TGFβ1	Differentiation, Invasion	64, 65

4.2 Limitations

This study contains a number of inherent limitations. Differential expression analysis in this study is conducted in comparison to gestational age-matched controls. Although it is beneficial to compare placental samples of similar gestational age because of the prime consideration of comparing placental tissues that are of similar developmental stages, controls in this study are from preterm births that may not be considered normal nor healthy since evidence shows that preterm births are a result of perturbations during gestation some of which can be attributed to the placenta. In the future, inclusion of both preterm and term controls may be beneficial for understanding how selection of control samples impacts results. Inclusion of labored and non-labored controls is also important to further investigate impact of labor on placental miRNA and gene expression. Due to sample scarcity and time limitations, a secondary validation cohort was not included in

this study. Including a discovery and validation cohorts is beneficial to ensure that findings are replicable, concrete, and independent of any experimental biases.

Analysis in this study also assumes that cellular compositions across tissue samples is equal however that is likely untrue, even though we conducted multi-site placental sampling focused solely on the fetal side of the placenta. The placenta contains a mosaic of cell types that can exist at varying ratios due to inherent variability between patient samples or variability in tissue sampling. To resolve this limitation, fast and effective methods for isolation of different cell types within the placenta are necessary. In addition to the importance of establishing transcriptional profiles in order to identify contributions of each cell type to the placental transcriptional landscape. Finally, as with all investigations of delivered placental samples, it is impossible to delineate whether observed molecular differences are part of the cause or the consequence of disease.

4.3 Future Studies

There are many future studies that can stem from this work. Since miRNAs are known to enter maternal circulation during pregnancy, one important future study would be to measure miRNA levels in maternal plasma of patients diagnosed with PE and/or IUGR to assess miRNA diagnostic potential, particularly miRNAs identified in all three patient groups. More recent research demonstrates the potential use of placental exosomes as biomarkers with standardization of isolation methods and identification of placenta specific markers for exosomes.[66,67] Some miRNAs belonging to the C19MC have been detected in exosomes from primary trophoblast cells.[66] Another marker is *PLAP*

(Placental Alkaline Phosphatase) present on exosomes only in the peripheral circulation of pregnant women.[67] *PLAP* is mainly produced from SCT cells but has also been identified in other trophoblast cell types in the placenta.[68,69] For a future study, analysis of maternal plasma samples for exosomes or cell-free RNA can be completed retrospectively by measuring miRNA levels in plasma samples obtained early in pregnancy as a part of routine clinical care to more accurately assess predictive value across gestation.

Findings from global expression studies in the placenta can also be coupled with other clinical and histopathological information to better understand trophoblast dysfunction contributing to poor placental growth and function in PE and IUGR pregnancies. Finally, identification and validation of miRNA gene targets is an important future study to continue to expand our understanding of miRNAs and their role as epigenetic regulators in the placenta. Integration of multi-level molecular information combined with *in vitro* techniques is a useful approach. However, investigating individual miRNAs using targeted approaches combined with target prediction software and *in vitro* techniques is equally effective. *In vitro* techniques can also include assessing the impact of hypoxia on miRNA and gene target expression, since the intrauterine environment in PE and IUGR pregnancies is thought to be hypoxic.

4.4 Conclusion and Significance

In conclusion this study identified miRNAs and genes differentially expressed in three patient groups with early-onset pregnancy complications (PE only, IUGR only, and PE +

IUGR), and further investigated the potential impact of miRNAs on gene target expression and biological functions important for trophoblast cells of the placenta (figure 4.1). This study expanded our knowledge of molecular changes in the placenta in each patient group by identifying miRNAs and genes that are common and unique to each pregnancy complication. Additionally, this study provided further evidence for the role of miRNAs and their impact on placental growth/development and function.

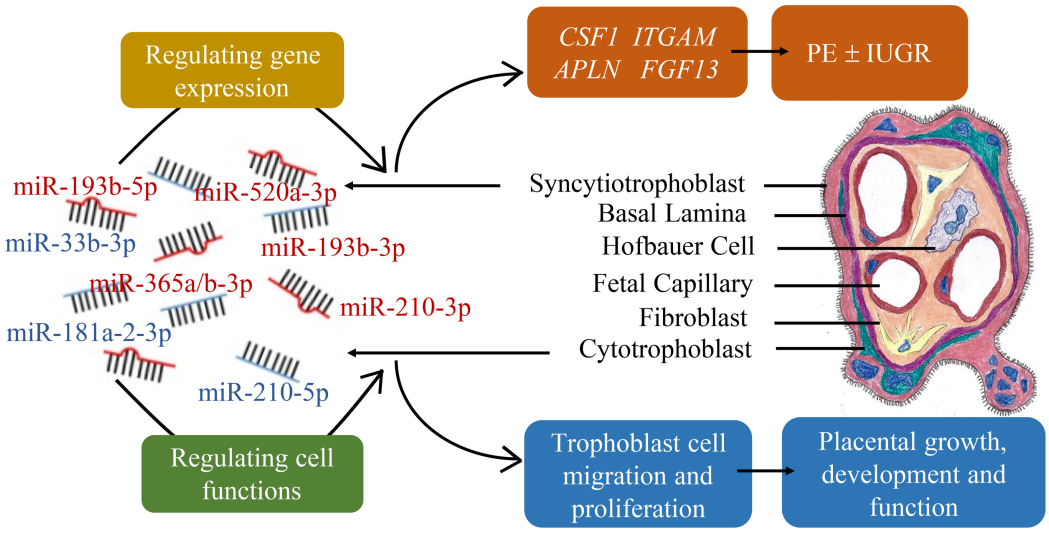

Figure 4.1 MicroRNAs impact gene expression and biological functions

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Appendix

Appendix A. Human Ethics Approval.

Research Western

Use of Human Participants - Ethics Approval Notice

Principal Investigator: Dr. Victor Han
File Number:102621
Review Level: Delegated
Approved Local Adult Participants:400
Approved Local Minor Participants:0
Protocol Title: Tissue and Blood Collection for Molecular Mechanisms of Preeclampsia
Department & Institution: Schulich School of Medicine and Dentistry\Anatomy & Cell Biology;Western University
Sponsor: Canadian Institutes of Health Research

Ethics Approval Date: June 12, 2012 **Expiry Date:**May 31, 2017
Documents Reviewed & Approved & Documents Received for Information:

Document Name	Comments	Version Date
Western University Protocol		
Letter of Information & Consent	Control Subjects - Version 1	
Letter of Information & Consent	Study Subjects - Version 1	

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the University of Western Ontario Updated Approval Request Form.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

The Chair of the HSREB is Dr. Joseph Gilbert. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

[redacted]
Signature

Ethics Officer to Contact for Further Information

[redacted]	[redacted]	[redacted]

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Curriculum Vitae

Name:	Zain Awamleh
Post-secondary Education and Degrees:	Doctorate of Philosophy, Department of Biochemistry, Collaborative with Developmental Biology, The University of Western Ontario, London, Ontario, Canada September 2013-April 2019 Bachelor of Science, Honors Specialization in Genetics The University of Western Ontario London, Ontario, Canada 2009-2013 HBSc.
Research Experience:	Clinical Research Assistant, Laboratory of Dr. Victor Han, Lawson Health Research Institute 2014-2018 Undergraduate Research Assistant/ Thesis Student, Laboratory of Dr. Shiva Singh, The University of Western Ontario 2011-2013 Undergraduate Research Assistant, Laboratory od Dr. Chil-Yong Kang, The University of Western Ontario 2012-2013
Related Work Experience:	Teaching Assistant, Biology 1001/1201, Department of Biology The University of Western Ontario 2017-2018 Teaching Assistant, Biochemistry 3380G, Department of Biochemistry, The University of Western Ontario 2014-2018
Awards:	1st Place Poster Presentation, CIHR-IHDCYH Award of Excellence, 6th National Perinatal Meeting, Mont Trémblant, Québec (2019) 1st Place Oral Presentation, Paul Harding Research Day, Department of Obstetrics and Gynecology, The University of Western Ontario (2018)

Conference Travel Award, Department of Paediatrics, The University of Western Ontario (2017)

2nd Place Oral Presentation, Paediatrics Research Day, Department of Paediatrics, The University of Western Ontario (2017)

1st Place Oral Presentation, CIHR-IHDCYH Award of Excellence, 4th National Perinatal Meeting, Montebello, Quebec (2017)

1st Place Poster Presentation, Paediatrics Research Day, Department of Paediatrics, The University of Western Ontario (2015)

Scholarships:

Paediatrics Graduate Scholarship, The University of Western Ontario $17,000 (2016)

Epigenetics Trainee Scholarship, Children's Health Research Institute, The University of Western Ontario $10,000 (2016)

Schulich Graduate Scholarship, Schulich School of Medicine and Dentistry, The University of Western Ontario $ 8000 (2015-2018)

Western Graduate Research Scholarship, The University of Western Ontario $24,000 (2014-2018)

Publications:

Awamleh Z, Gloor GB, Han VKM. "Placental microRNAs in pregnancies complicated with intrauterine growth restriction and preeclampsia: Potential impact on gene expression". Accepted to BMC Medical Genomics March 6th, 2019

Kim GN, Wu K, Hong JP, **Awamleh Z**, Kang CY. "Creation of matrix protein gene variants of two serotypes of vesicular stomatitis virus as prime-boost vaccine vectors". J Virol. 2015. 89(12): 6338-51.

Castellani CA, Melka MG, Wishart AE, Locke ME, **Awamleh Z**, O'Reilly RL, Singh SM. "Biological relevance of CNV calling Methods using familial relatedness including monozygotic twins." BMC Bioinformatics. 2014. 15: 114.

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